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Walia

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(54) **MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF**

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C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC *C12Q 1/66* (2013.01); *C12N 9/0069* (2013.01)

(58) **Field of Classification Search**
USPC 435/8
See application file for complete search history.

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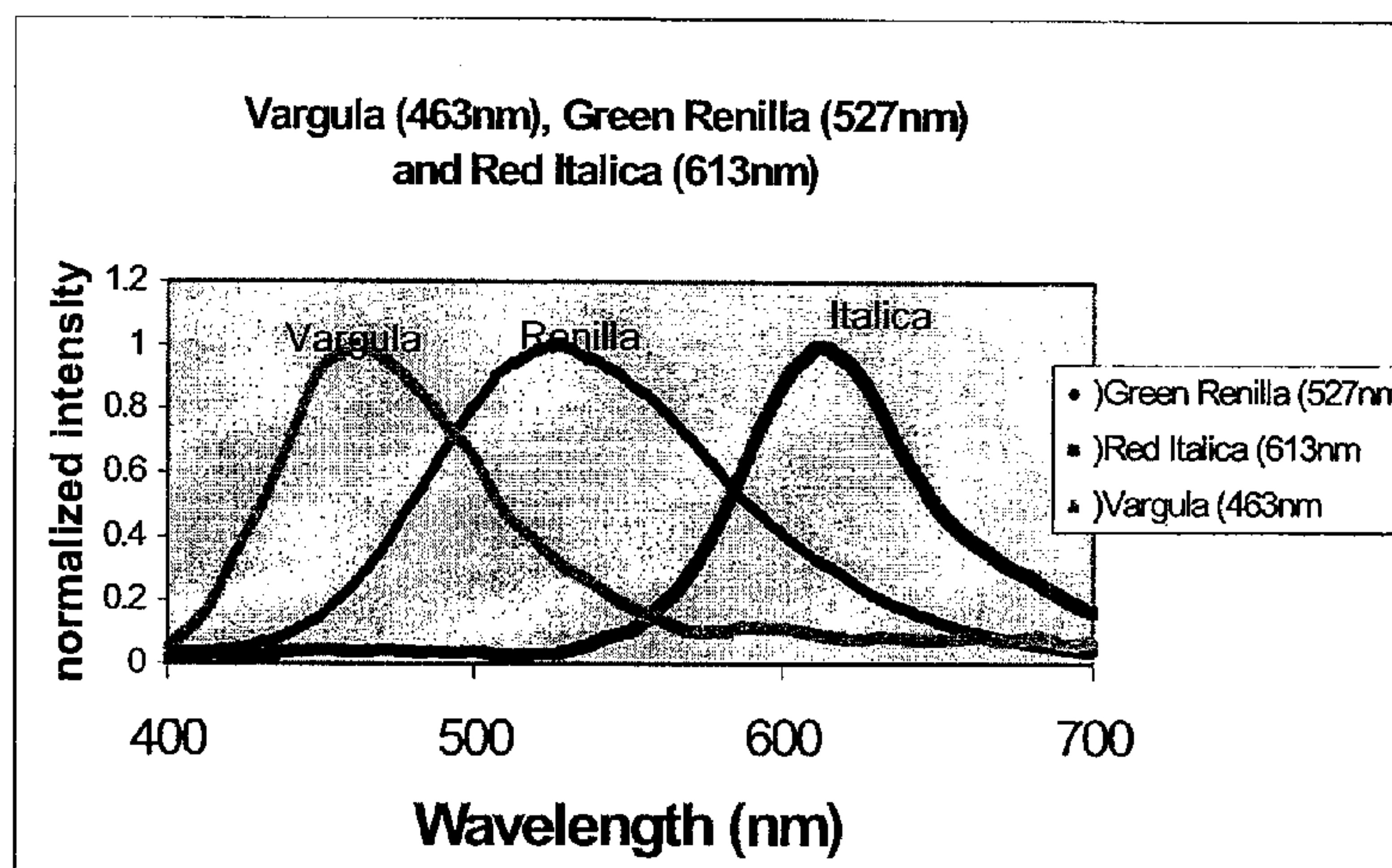
(Continued)

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(57) **ABSTRACT**

The present invention encompasses modified luciferases, methods for making modified luciferases, and assays utilizing modified luciferases. Modified luciferases of the invention show increased activity over wildtype luciferases and also show increased stability of signal. The present invention also encompasses multiplex assays utilizing multiple luciferases reporters with different emission spectra and different substrates for simultaneous luciferase measurements.

1 Claim, 42 Drawing Sheets



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FIG. 1

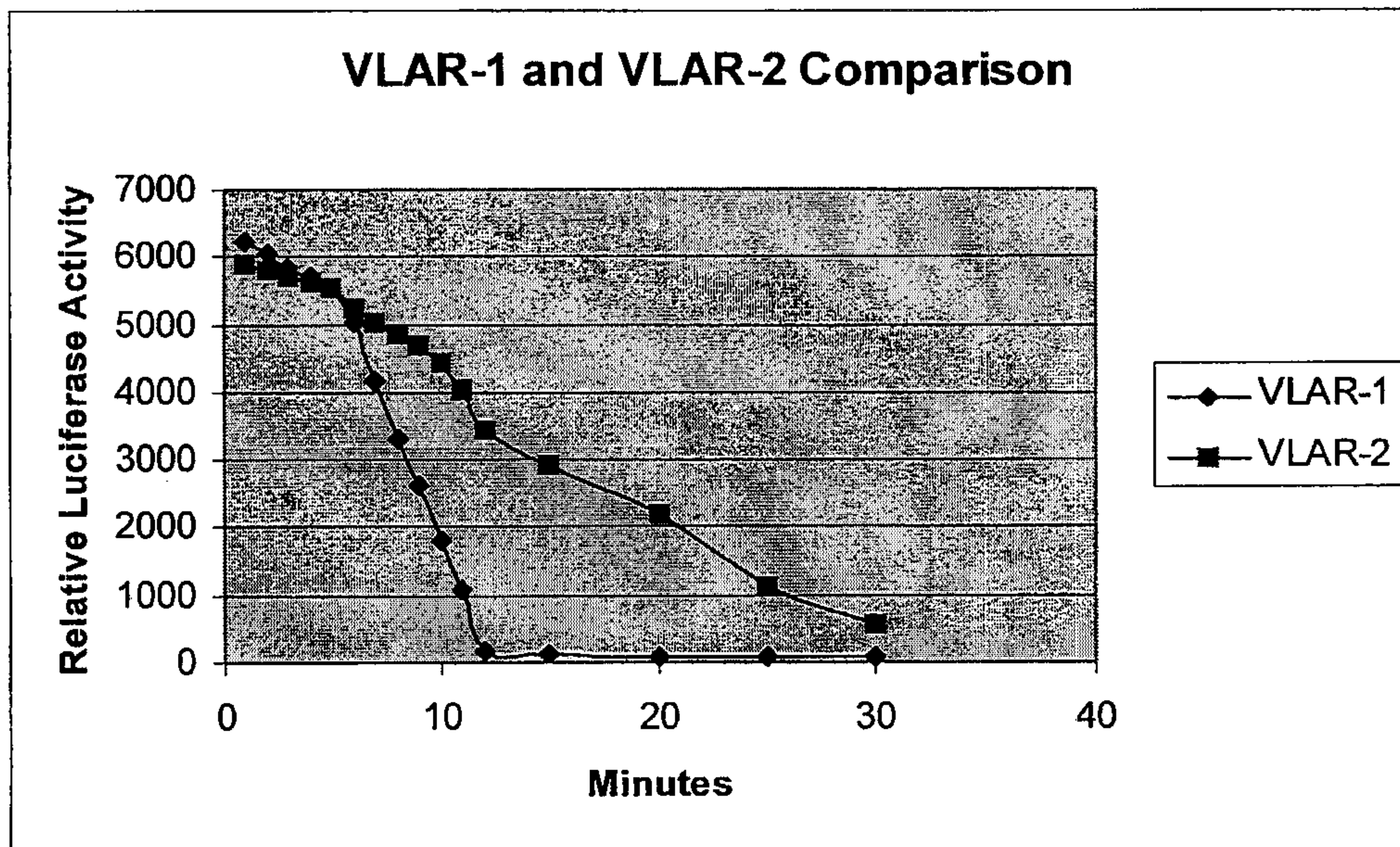


FIG. 2A. Sample volume 20 μ l

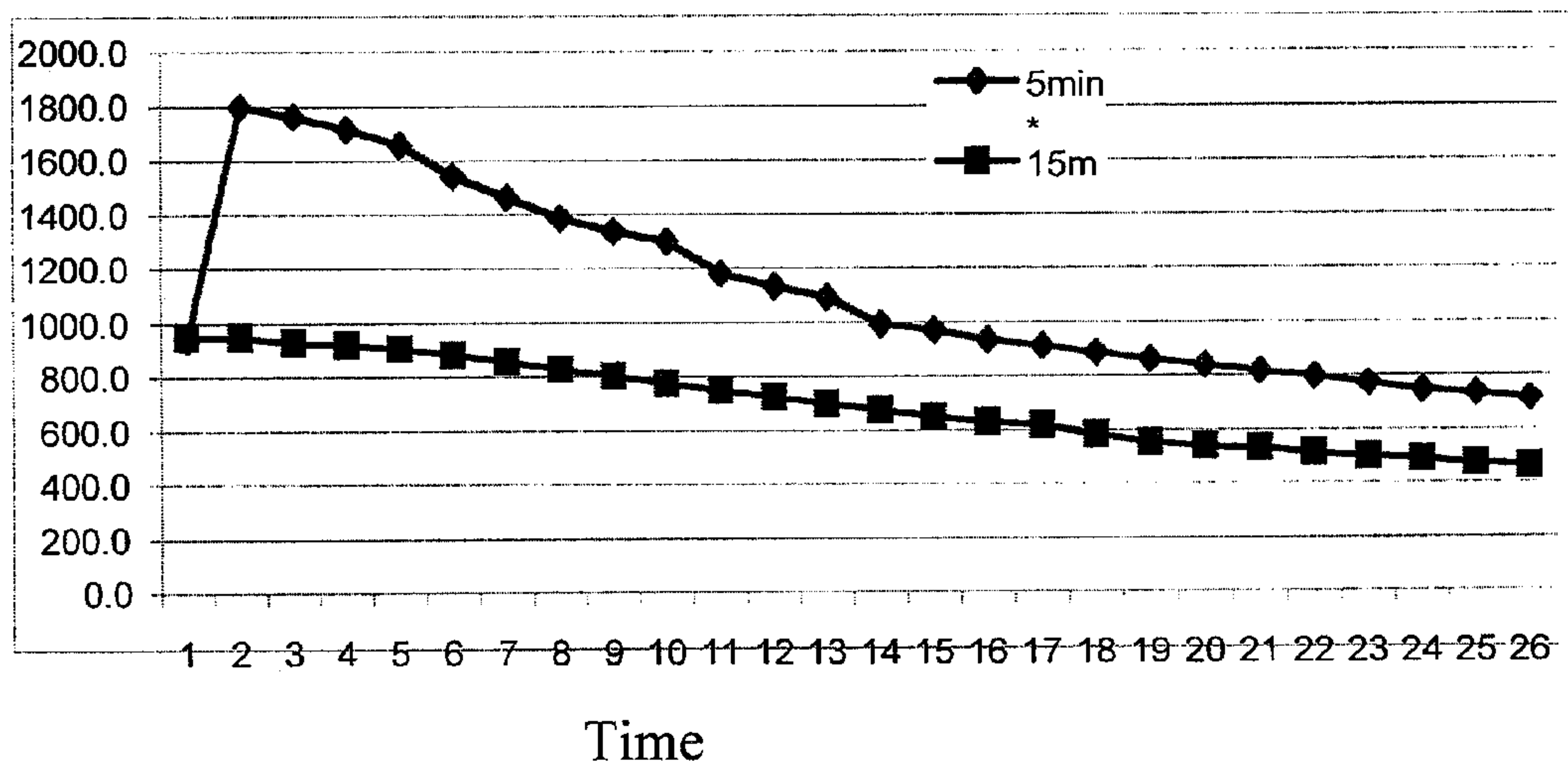


FIG. 2B. Sample volume 5

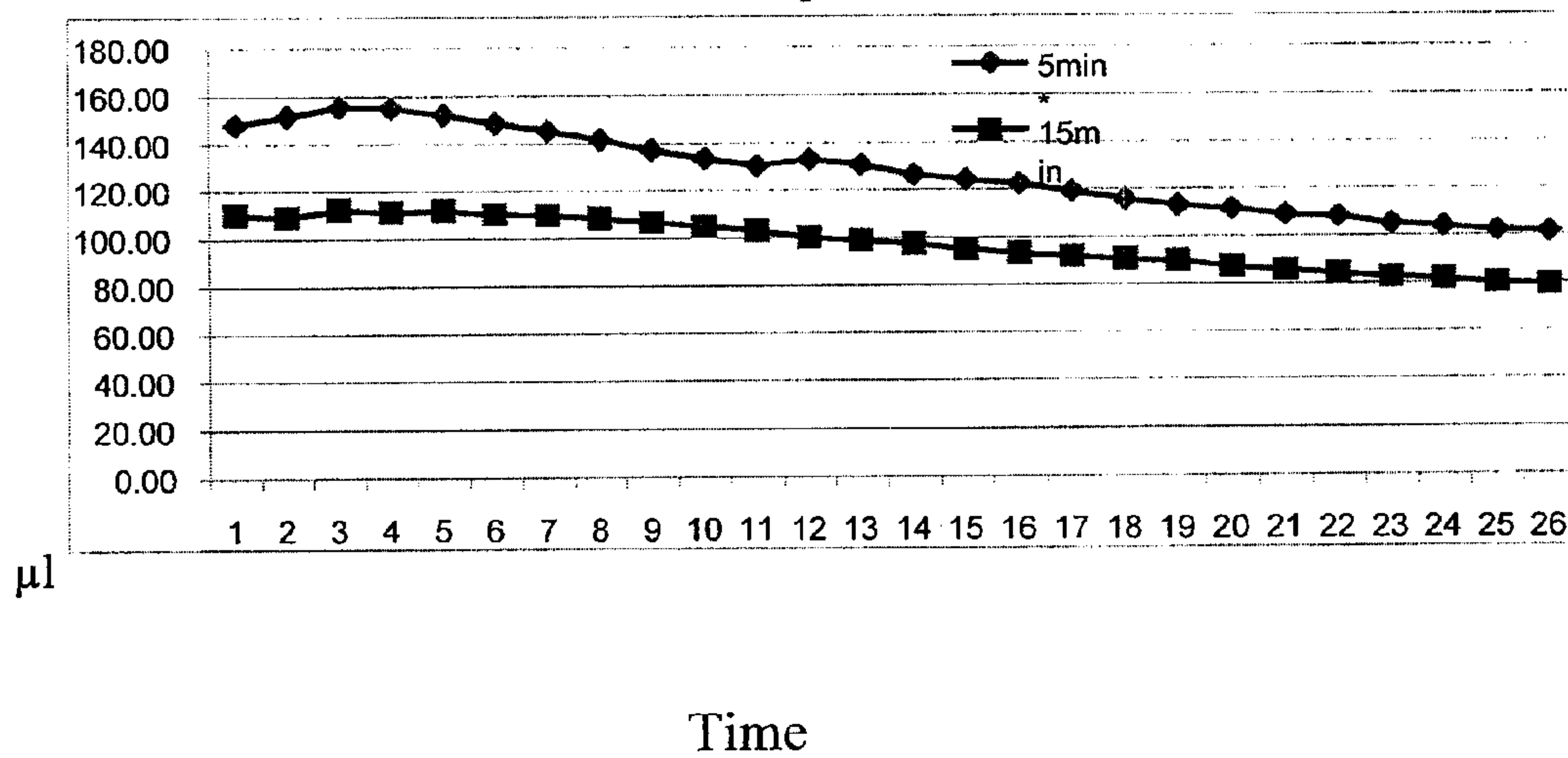


FIG. 3A

pCMV Green Renilla Luciferase plasmid Sequence (SEQ ID NO: 1):

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggctgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagtattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagtccg cgttacataa ctacggtaa atggcccgcg tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgatg ttcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggg aaactgcccc ctggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggacttcc ctacttggca gtacatctac gtattagtca
601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661 actcacgggg attccaagt ctccacccca ttgacgtcaa tgggagtttg tttggcacc
721 aaaatcaacg ggactttcca aaatgtcgt acaactcgc cccattgacg caaatgggcg
781 gtaggcgtgt acgggtggag gtctatataa gcagagctct ctggctaact agagaacca
841 ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gcttggtagc
901 gagctcggat ccatgtgtt gaaagtgtg tttgctattg gatgtatcgt agtgcaggct
961 atggcctcaa aagtgtacga tccggagcag cggaagagga tgatcacggg gccccaatgg
1021 tgggcacgat gcaagcagat gaatgtgtt gacagttca ttaactacta cgacagcgag
1081 aaacacgcgg agaacgcagt gatattcctg cacggcaatg caaccagtag ctatctgtgg
1141 agacacgtgg tgccicatat tgagccggtc gctagatgca ttattcccga tcttattgga
1201 atgggaaat ccggaagag tggaaatgga tcatatagcc tctcogatca ttataaatat
1261 ctgactgctt ggttgaatt gctcaatctg cccaagaaaa tcatcttgt aggacatgat
1321 tggggctccg ccctgctt tcaattatgcc tatgaacacc aggatcggat caaggctatt
1381 gttcacatgg agagcgtgtt ggatgtgat gaatcatgga tgggtggcc ggatatagaa
1441 gaagagctgg cgctgattaa atctgaggag ggcgagaaga tggactcga aaataacttc
1501 ttgtcgaga cggactgcc cagtaagatc atgcgcaaac tggagcctga agagtttgcg
1561 gcttacctgg aaccctcaa ggagaaggga gaggtgagga gaccgacct gcatggcct
1621 cgggaaatc cgctggtaa aggaggaag ccagacgtcg tcgccattgt ccggaattac
1681 aacgctacc tccgcgctag tgacgacctg cctaaactct tcatcgaatc agatcctgtt
1741 ttcttagta acgcatcgt cgagggcgcc aagaagttc caaacaccga attgttaaa
1801 gtcaaaggac ttacttctt ccaggaggat gcgcccgatg aaatgggaaa gtatatcaaa
1861 tcctcgtgg agaggtctt gaagaatgag cagaggtcca tctagtctag aaataattct
1921 tactgtcatg ccaagtaaga tgctttctg tgctgcaata gcaggcatgc tgggatgcg
1981 gtgggctcta tggctctga ggcgaaaga accagctggg gctctagggg gtatccccac
2041 gcgcccgtg gcggcgcat aagcgcggcg ggtgtgtgg ttacgcgcag cgtgaccgct
2101 acactgcca gcgccctagc gcccgctct tctgcttct tcccttctt tctcggcag
2161 ttcgcccgt tccccgtca agctctaaat cggggcatcc cttaggggt cggattagt
2221 gctttacggc acctcgacc caaaaaact gattaggggt atggttcacg tagtgggcca
2281 tcgcccgtat agacggttt tcgcccctt acgttggagt ccacgttct taatagtgga
2341 ctctgttcc aaactggaac aacctcaac cctatctgg tctattctt tgattataa
2401 gggatttgg ggattcggc ctattggta aaaaatgagc tgatttaaca aaaattaac
2461 gcgaattaat tctgtggaat gtgtgtcagt taggtgtgg aaagtccca ggctccccag
2521 gcaggcagaa gtatgcaag catgcatctc aatagtcag caaccaggtg tggaaagtcc
2581 ccaggctccc cagcaggcag aagtatgca agcatgcatc tcaattagtc agcaaccata
2641 gtcccggccc taactccgc catcccggc ctaactccgc ccagttccgc ccattctccg
2701 ccccatggct gactaattt tttatttat gcagaggccg aggcgcctc tgcctctgag
2761 ctattccaga agtagtgagg aggttttt ggaggcctag gctttgcaa aaagctcccg
2821 ggagctgta tatccattt cggatctgat caagagacag gatgaggatc gtttcgcatg
2881 attgaacaag atggattgca cgcaggtct ccggccgctt ggggtgagag gctattcggc
2941 tatgactggg cacaacagac aatcggctgc tctgatgccg ccgtgttccg gctgtcagc
3001 caggggcgcc cggttcttt tctcaagacc gacctgccc gtgccctgaa tgaactgcag
3061 gacgaggcag cgcggctatc gtggctggc acgacgggcg ttccttgcgc agctgtgctc
3121 gacgtgtca ctgaagcggg aaggactg ctgctattg gcgaagtgc gggcaggat

FIG. 3B

3181 ctctgtcat ctcacctgc tctgcccag aaagtatcca tcatggctga tgcaatgagg
3241 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatcgcatc
3301 gagcagcac gtactcggat ggaagccggc ctgtcgcac aggatgatct ggacgaagag
3361 catcaggggc tcgcccagc cgaactgtc gccaggctca aggcgcgcac gcccgacggc
3421 gaggatctcg tcgtgacca tggcgatgcc tgcctgccga atatcatggt ggaaaatggc
3481 cgctttctg gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
3541 gcgttggcta cccgtgatat tgctgaagag ctggcggcg aatgggctga ccgcttcctc
3601 gtgcttacg gtatcgccgc tcccgatcgc cagcgcacgc ccttctatcg ccttctgac
3661 gagttctct gagcgggact ctggggctcg aatgaccga ccaagcgcgc cccaacctgc
3721 catcacgaga ttctgattcc accgcccct tctatgaaag gttgggctc ggaatcggtt
3781 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttctcgccc
3841 acccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcaciaaatt
3901 tcaciaataa agcattttt tctctgact ctagtgtgg ttgtccaaa tcatcaatg
3961 tctctatca tctctgtata ccgtcgacct ctactagag ctggcgtaa tcatggctat
4021 agctgttcc tgtgtgaaat tcttatccgc tcacaattcc acacaacata cgagccggaa
4081 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc
4141 gctcactgcc cgcttccag tcgggaaacc tctcgtgcca gctgcattaa tgaatcggcc
4201 aacgcgcggg gagagggcgt ttgcgtattg ggcgctctc cgcttctcg tctactgact
4261 cgctgcgctc ggtcgtcgg ctgcggcagc cggatcagc tctcctcaag gcggaatac
4321 gggtatccac agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
4381 agccaggaa ccgtaaaaag gccgcgttc tggcgtttt ccataggctc cccccctg
4441 acgagcatca caaaaatcga cgctcaagtc agaggtggc aaaccgcaca ggactataaa
4501 gataccaggc gttccccct ggaagctccc tctgctcgc tctgttccg accctgccc
4561 ttaccggata cctgtccgc tttctcctt cgggaagcgt ggcgcttct caatgctac
4621 gctgtaggta tctcagttc gtgtaggctg tctcctcaa gctgggctgt gtgcacgaac
4681 cccccgtca gcccgaccgc tgcgcctat ccgtaacta tctcttgag tccaaccgg
4741 taagacacga ctatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt
4801 atgtaggcgg tctacagag tcttgaagt ggtggcctaa ctacggctac actagaagga
4861 cagtattgg tctcgcgt ctgctgaagc cagtacctt cggaaaaaga gttgtagct
4921 ctgatccgg caaacaacc accgctggtg gcggtggtt tttgttgc aagcagcaga
4981 ttacgcgcag aaaaaagga tctcaagaag atccttctat ctttctacg ggtctgacg
5041 tctagtggaa cgaaaactca cgttaagga tttggtcat gagattatca aaaaggatct
5101 tcacctagat cttttaaatt taaaaatgaa gtttaaact aatctaaagt atatagat
5161 aaacttggc tgacagttac caatgctaa tctagtggc acctatctca gcatctgtc
5221 tattctgtc atccatagtt gctgactcc ccgtcgtgta gataactacg atacgggagg
5281 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccgctccag
5341 attatcagc aataaaccag ccagccggaa gggccgagc cagaagtgg cctgcaactt
5401 tatccgctc catccagctt attaatgtt gccgggaagc tagagtaagt agttcgcag
5461 ttaatagtt gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtct
5521 ttggtatggc tctatcagc tccggtccc aacgatcaag gcgagttaca tgatcccca
5581 tttgtgcaa aaaagcgggt agctcctcg gtcctccgat cgtgtcaga agtaagtgg
5641 ccgagtggt atcactcatg gttatggcag cactgcataa tctcttact gcatgccat
5701 ccgtaagatg ctttctgtg actggtgagt actcaaccaa gtcattctga gaatagtga
5761 tgcggcacc gagttgctt tcccggcgt caatacggga taataccgcg ccacatagca
5821 gaacttaaa agtgctcatc attgaaaac gttctcggg gcgaaaactc tcaaggatct
5881 taccgctgt gagatccagt tcatgtaac ccactcgtc acccaactga tctcagcat
5941 ctttactt caccagcgt tctgggtgag caaaaacagg aaggcaaat gccgcaaaa
6001 agggaataag ggcgacacgg aatgtgaa tactcact cttctttt caatattt
6061 gaagcattt tcaagggtt tctctatga gcgatacat attgaaatg attagaaaa
6121 ataaacaat aggggtccg cgcacattc cccgaaaagt gccacctgac gtc

FIG. 4A

Modified red firefly luciferase with secretory signal (SEQ ID NO: 2)

1 gacggatcgg gagatcctcc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggctgct gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301 tggagtccg cgttacataa ctacggtaa atggcccgcg tggctgaccg cccaacgacc
361 cccgccatt gacgtcaata atgacgatg ttccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tattacggg aaactgcca ctggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gctggcatt
541 atgccagta catgacctta tgggactttc ctactggca gtacatctac gtattagta
601 tcgctattac catgggtgat cggtttggc agtacaatca tgggcgtgga tagcggttg
661 actcacgggg atttccaagt ctccaccca ttgacgtcaa tgggagttg tttggcacc
721 aaaatcaacg ggactttcca aaatgtcgt acaactccgc cccattgacg caaatggcg
781 gtaggcgtgt acgggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841 ctgctactg gcttatgaa ataatagca ctactatag ggagaccaa gcttggtacc
901 gagctcggat cc atggccttctctgtggctgctgtcctgctgggacctgctgggcaccaccttccggc
961 taccgatcg aggagggtc tgcggcctc caattgcaca agtacaatgca acaatcgcg
1021 aagctcggcg ccatcgctt cagtaacgcc ctgacaggcg tcgacaatcag ctaccagcag
1081 tacttcgaca tcacgtgcag actcggcag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagtct tcaattctgt tctggctggt
1201 cttacatcg gagttacgt cgcgccaact aacgaaatt atacactag agagctgaac
1261 cacagtctgg ggatagcca acctactatc gtattctca gcaggaagg cctgccc aaa
1321 gtgctgagg tgcagaagac cgtgacttgc atcaaaacca ttgtatcct ggacagtaag
1381 gtcaactcg ggggtatga ctgcgtagag acctcatta agaaacacgt cgagctgggc
1441 tttctgcca cctcattgt gccatcgac gtcaaaagacc ggaagacca cttgctctg
1501 cttatgaact ctccgggtc cacagggtc ccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctca cgtaaggac cctatatac gcaatcaggt ggcccaggt
1621 accgctacc tgactgtgt gccttccac cacggctcg gaatgtcac tacttgggc
1681 tacttgcct gcggtaccg gattgtcat ctactaagt tcgacgagga gctttctg
1741 cgcacactc aggattaca gtgcactaca gtaatcctgg tccgacact gttcgcaatt
1801 ctaataggt ctgagctct tgataagtt gacctcta acctgactga aatagccagc
1861 ggtggtgct cactgcca ggagatcggc gaggtgtg caagaagatt caacctcca
1921 ggcgtccggc agggatatg actcaccgag actaccagtg ctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgtcaaggi gaagattatt
2041 gacctgata ccaagaaaac gttgggtgc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctcta tgtgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca ctcttata
2221 gtcgaccgcc tgaatctct cattaagat aaaggatacc aagtccacc agctgaactg
2281 gactctgtc tctgcaaca ccctaacatt agagatgctg gtgtggccg ggtcccagc
2341 agcagggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tcgtagacta tgtaaactcc caggtgtca accacaagcg gctgaggggc
2461 ggcgtcggg tcgtagatga agtcccaag ggcctcacag gaaagatcga cgcgaaagt
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctgatctag aaataattc
2581 tactgtcatg ccaagtaaga tctttctg tgctgcaata gcaggcatgc tgggatgagc
2641 gtgggctca tggcttctga ggcgaaaga accagctggg gctctagggg gtatcccac
2701 gcgcccgtg gcggcgcat aagcgcggcg ggtgtggtg ttacgcgag cgtgaccgct
2761 acactgcca gcgcctagc gccgctct tctgcttct tccctctt tctgcccag
2821 ttcgcccgt tccccgta agctctaaat cggggcatcc cttagggtt ccgattagt
2881 gcttacggc acctcgacc caaaaaact gattaggggt atggitcacg tagtgggcca
2941 tgcctgat agacggttt tgcctttg acgtggagt ccacgtctt taatagtga
3001 ctctgtcc aaactggaac aacctcaac cctatctcg tctattctt tgattataa
3061 gggatttgg ggattcggc ctattgtta aaaaatgagc tgatttaaca aaaattaac
3121 gcgaattaat tctgtggaat gtgtgtagt taggtgtg aaagtccca ggctcccag
3181 gcaggcagaa gtatgcaag catgcatctc aatagtcag caaccagggt tggaaagtcc

FIG. 4B

3241 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata
3301 gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg
3361 ccccatggct gactaatttt ttttaattat gcagaggccg aggccgcctc tgcctctgag
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttgcaa aaagctcccc
3481 ggagcttcta tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggtgagag gctattcggc
3601 taigactggg cacaacagac aatcggctgc tctgatgccg ccgtgtccg gctgtcagcg
3661 cagggcgcc cggttcttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttcttgccg agctgtgctc
3781 gacgttgca ctgaagcggg aaggactgg ctgctattgg gcgaagtgc ggggcaggat
3841 ctctgtcat ctacctgc tctgccgag aaagtatcca tcatggctga tgcaatgcgg
3901 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatgcac
3961 gagcgagcac gtactcggat ggaagccggt ctgtcgcac aggatgatct ggacgaagag
4021 catcaggggc tgcgcccagc cgaactgttc gccaggctca aggcgcgcat gcccgacggc
4081 gaggatctcg tctgaccca tggcgatgcc tctgtccga atatcatggt ggaaaatggc
4141 cgctttctg gattcatga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
4201 gcgttgcta cccgtgatat tctgaagag ctggcggcg aatgggctga ccgcttctc
4261 gtgctttac gtatgccgc tcccattcg cagcgatcg cctctatcg ccttctgac
4321 gagtcttct gagcgggact ctggggttcg aatgaccga ccaagcgacg cccaacctgc
4381 catcacgaga ttctgattcc accgccgct tctatgaaag gttgggcttc ggaatcggtt
4441 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttctcggccc
4501 accccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcacaat
4561 tcacaaataa agcattttt tcaactgact ctagtgtgg tttgtccaaa ctcatcaatg
4621 tatcttaca tctctgata ccgtcgacct ctactagag ctggcgtaa tcatggctat
4681 agctgttcc tgtgtgaaat tttatccgc tcacaattcc acacaacata cgagccggaa
4741 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttc
4801 gctcactgcc cgcttccag tccggaaacc tctctgcca gctgcattaa tgaatcggcc
4861 aacgcgcggg gagaggcgg ttcgctattg ggcgctctc cgcttctcg ctactgact
4921 cgctgcgctc ggtcgttcgg ctgcggcgag cggatcagc tcaactcaaag gcgtaatac
4981 gttatccac agaactcagg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
5041 agccaggaa ccgtaaaaag gccgcgttc tggcgtttt ccataggctc cgccccctg
5101 acgagcatca caaaaatcga cgctcaagtc agagggtggc aaaccgaca ggactataaa
5161 gataccaggc gttccccct ggaagctccc tctgctcctc tctgttccg acctgccc
5221 ttaccggata cctgtccgc ttctccctt cggaagcgt ggcgcttct caatgctcac
5281 gctgtaggta tctcagttcg gctaggctc tctgctcaa gctgggctgt gtgcacgaac
5341 ccccgctca gcccgaccgc tgcgcttat ccgtaacta tctcttgag tccaaccgg
5401 taagacagca cttatcgcca ctggcagcag ccactgtaa caggattagc agagcgagg
5461 atgtaggcgg tctacagag ttctgaagt gttggcctaa ctacggctac actagaagga
5521 cagtattgg taactgcgct ctgctgaagc cagtacctt cggaaaaaga gttgtagct
5581 ctgatccgg caaacaacc accgctgcta gcggtggtt tttgtttgc aagcagcaga
5641 ttacgcgcag aaaaaagga tctcaagaag atcctttgat ctttctacg gggctgacg
5701 ctactggaa cgaaaactca cgttaagga tttggtcat gagattatca aaaaggatct
5761 tcacctagat cttttaaata taaaaatgaa gttttaaata aatctaaagt atatatgagt
5821 aaactggtc tgacagtac caatgctaa tcaagtggc acctatcica gcgatctgtc
5881 tattcgttc atccatagt gctgactcc ccgtcgtgta gataactac atacgggagg
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag
6001 attatcagc aataaaccag ccagccggaa gggccgagcg cagaagtggc cctgcaact
6061 tatccgctc catccagtct ataatgtt gccgggaagc tagagtaagt agttcgccag
6121 ttaatagtt gcgcaacgtt gttgccattg ctacaggcat cgtggtgta cgctcgtct
6181 ttggtatggc ttactcagc tccggtccc aacgatcaag gcgagttaca tgatcccca
6241 tgttgtaaa aaaagcgggt agctccttcg gtcctccgat cgtgtcaga agtaagtgg
6301 ccgcagtgtt atcactcatg gttatggcag cactgcataa ttcttact gtcatgcat
6361 ccgtaagatg ctttctgtg actggtgagt actcaaccaa gtactctga gaatagtga
6421 tgcggcgacc gagtgcctt tcccggcgt caatacggga taatacggcg ccacatagca
6481 gaactttaa agtgctcatc atggaaaac gttctcggg gcgaaaactc tcaaggatct
6541 taccgctgtt gagatccagt tcatgtaac ccactcgtc acccaactga tctcagcat

FIG. 4C

6601 ctttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa
6661 agggaataag ggcgacacgg aaatgtgaa tactcact cttcctttt caatattatt
6721 gaagcatta tcagggttat tgtctcatga gcggatacat attgaaatgt attagaaaa
6781 ataaacaaat aggggttccg cgcacattc cccgaaaagt gccacctgac gtc

FIG. 5

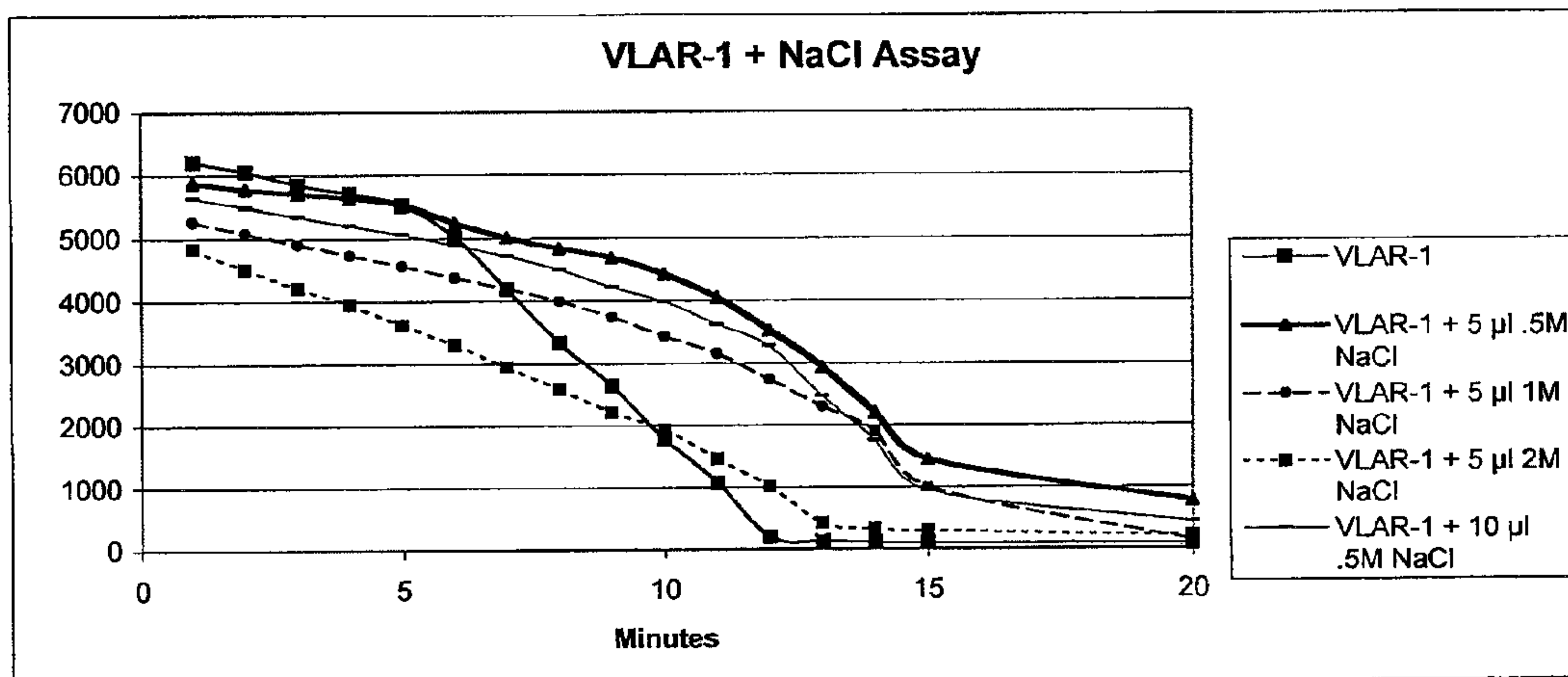


FIG. 6

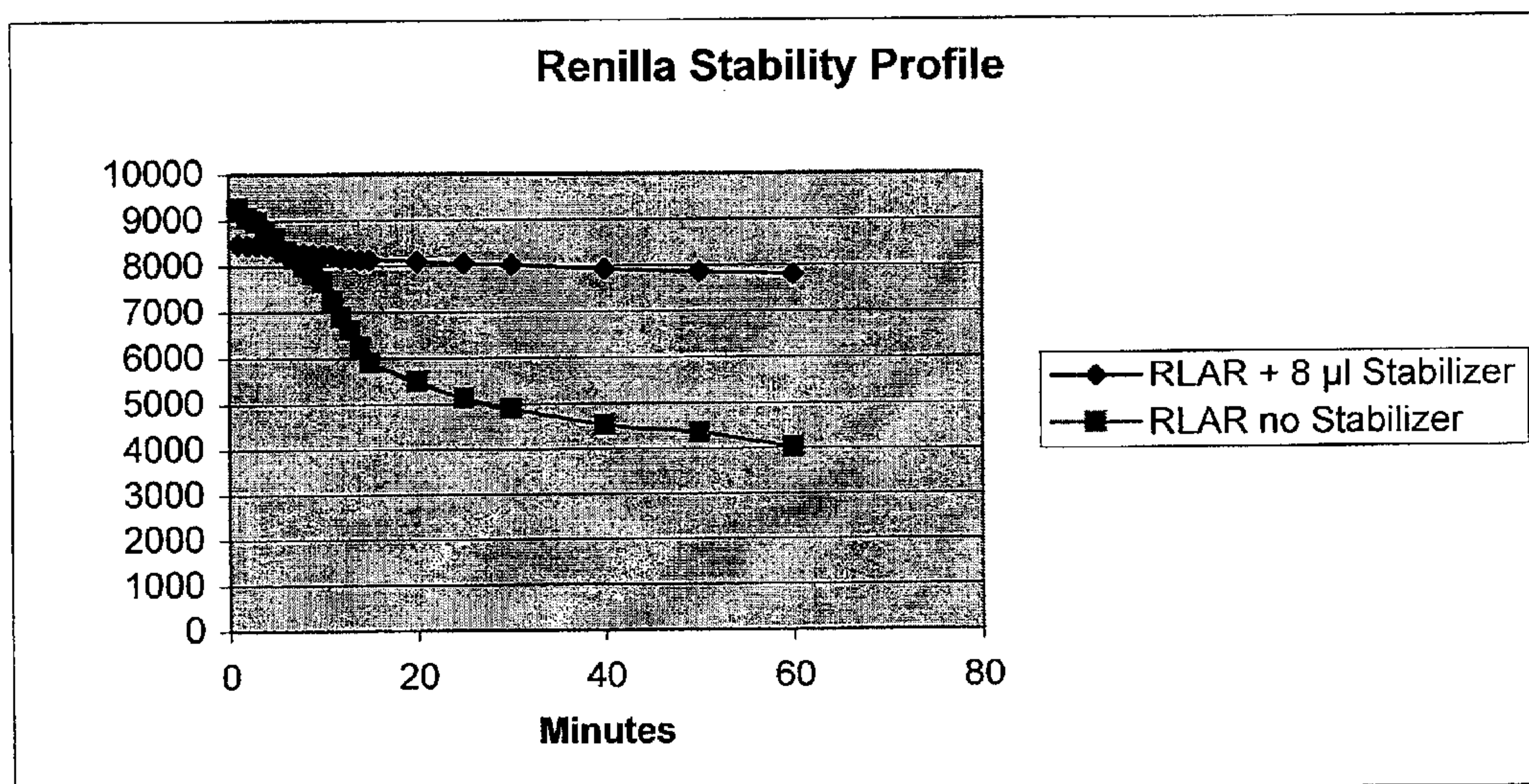


FIG. 7

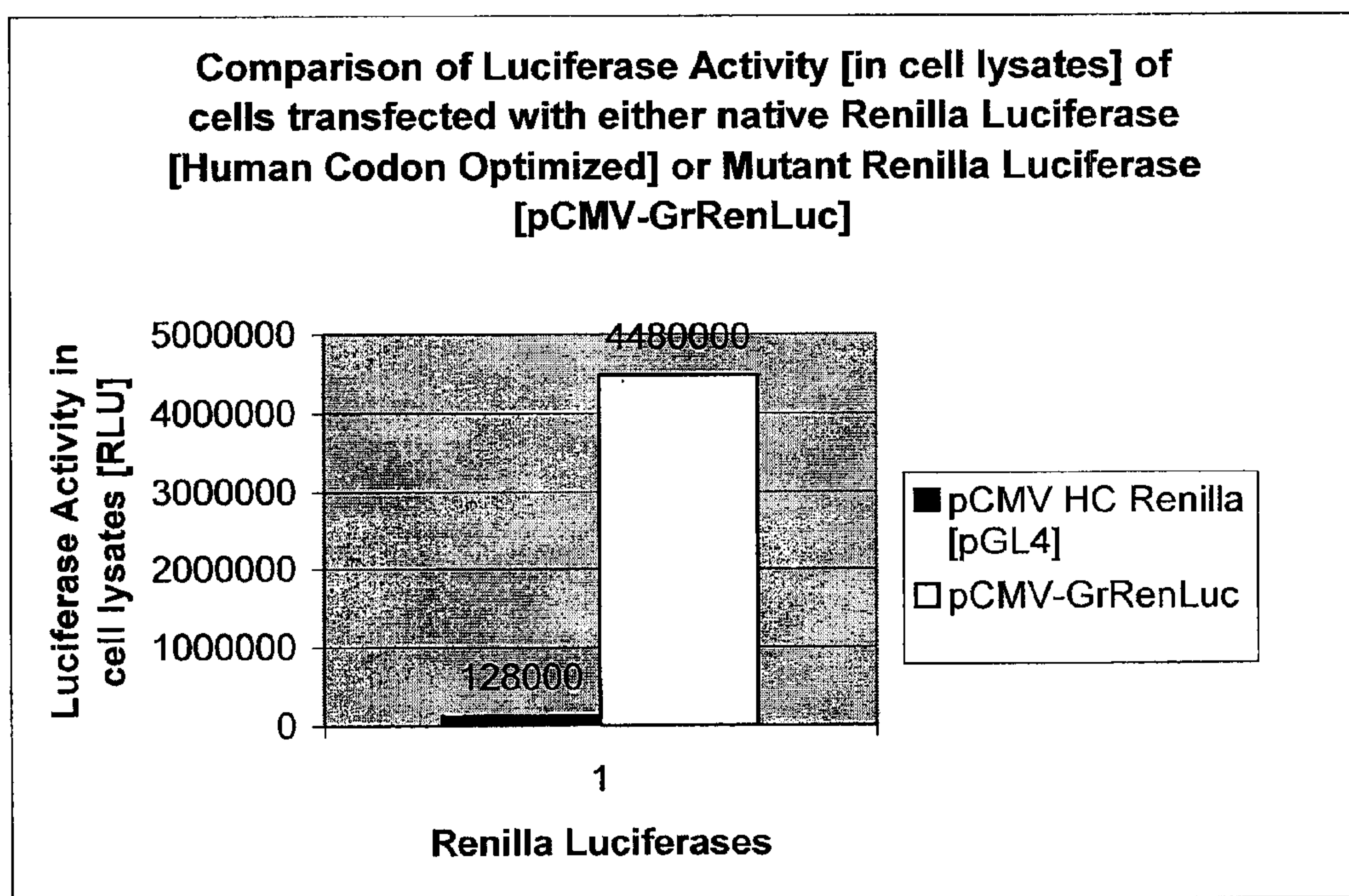


FIG. 8

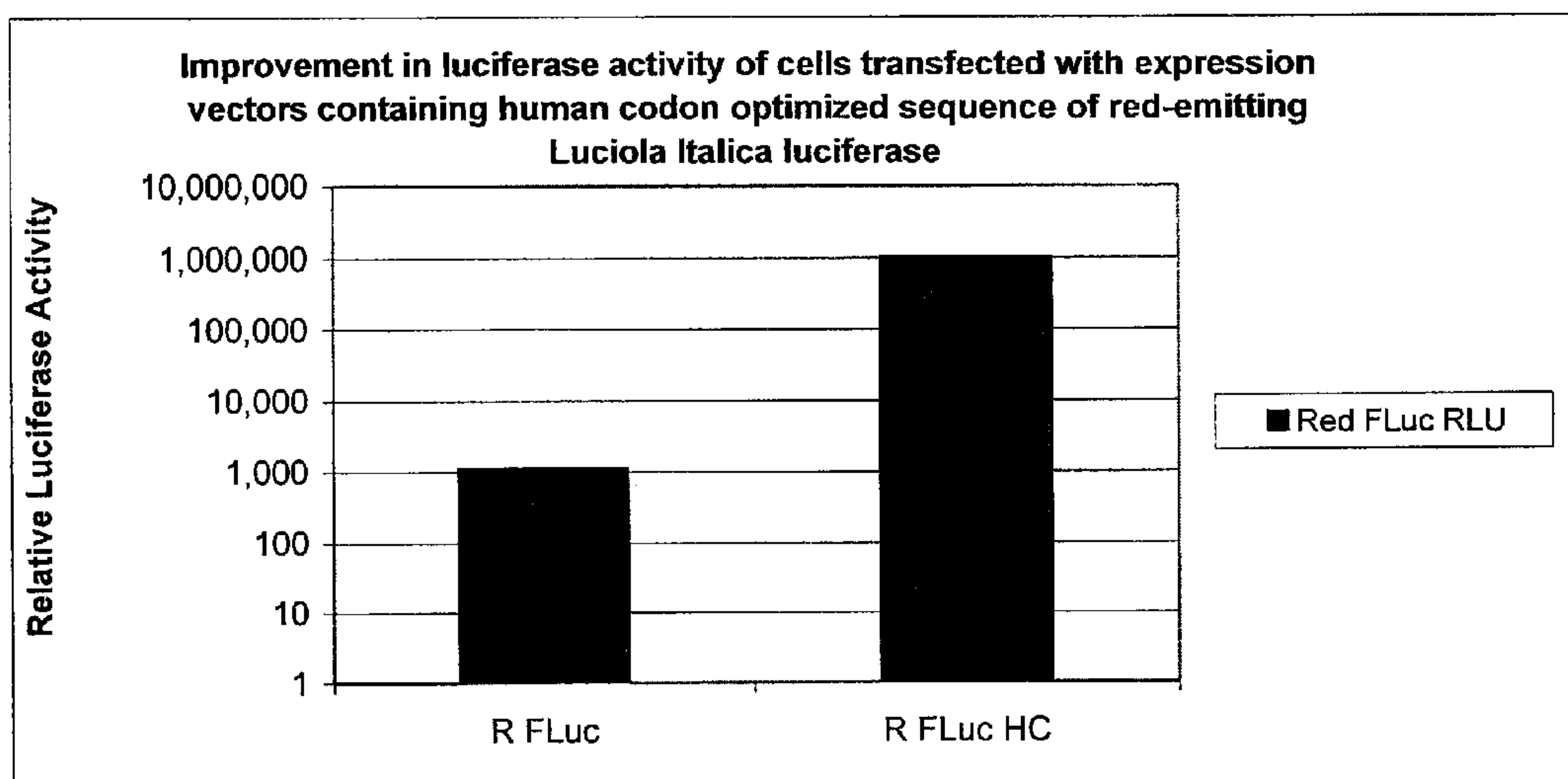


FIG. 9

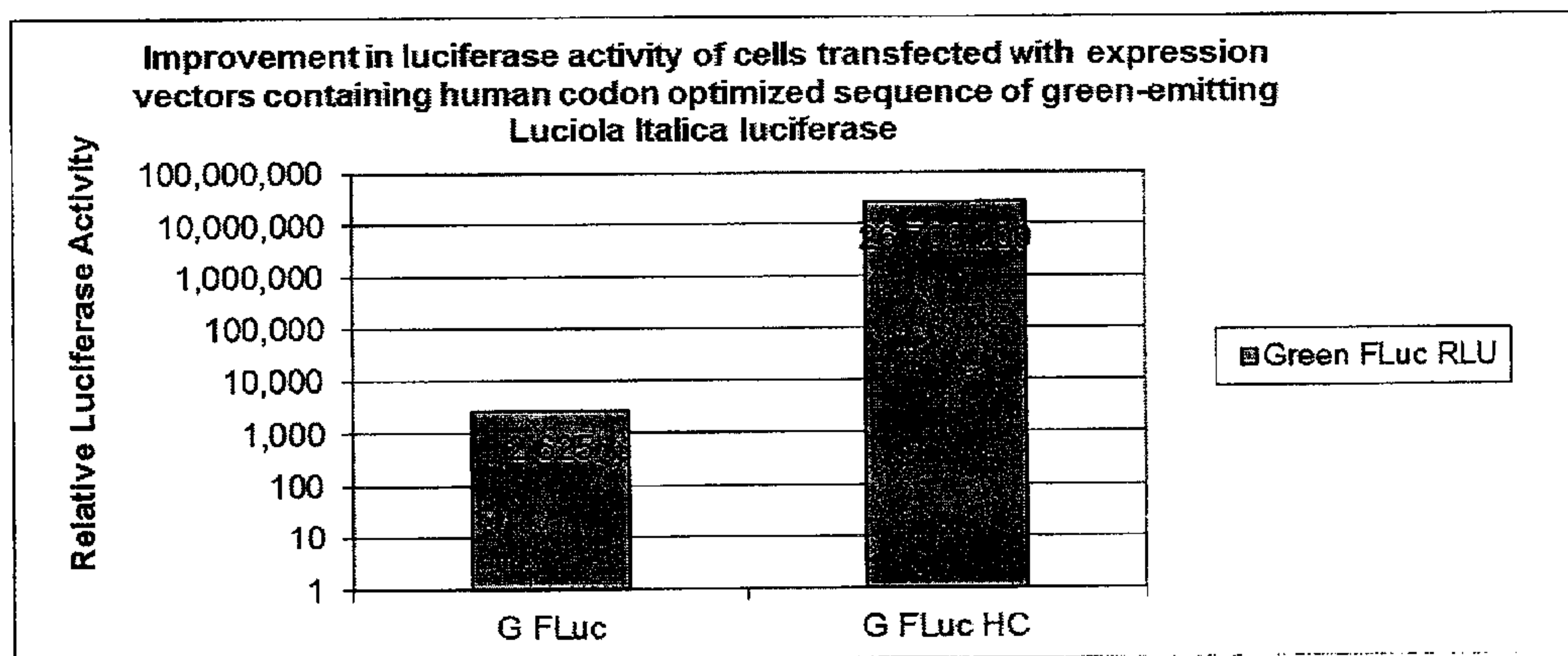


FIG. 10

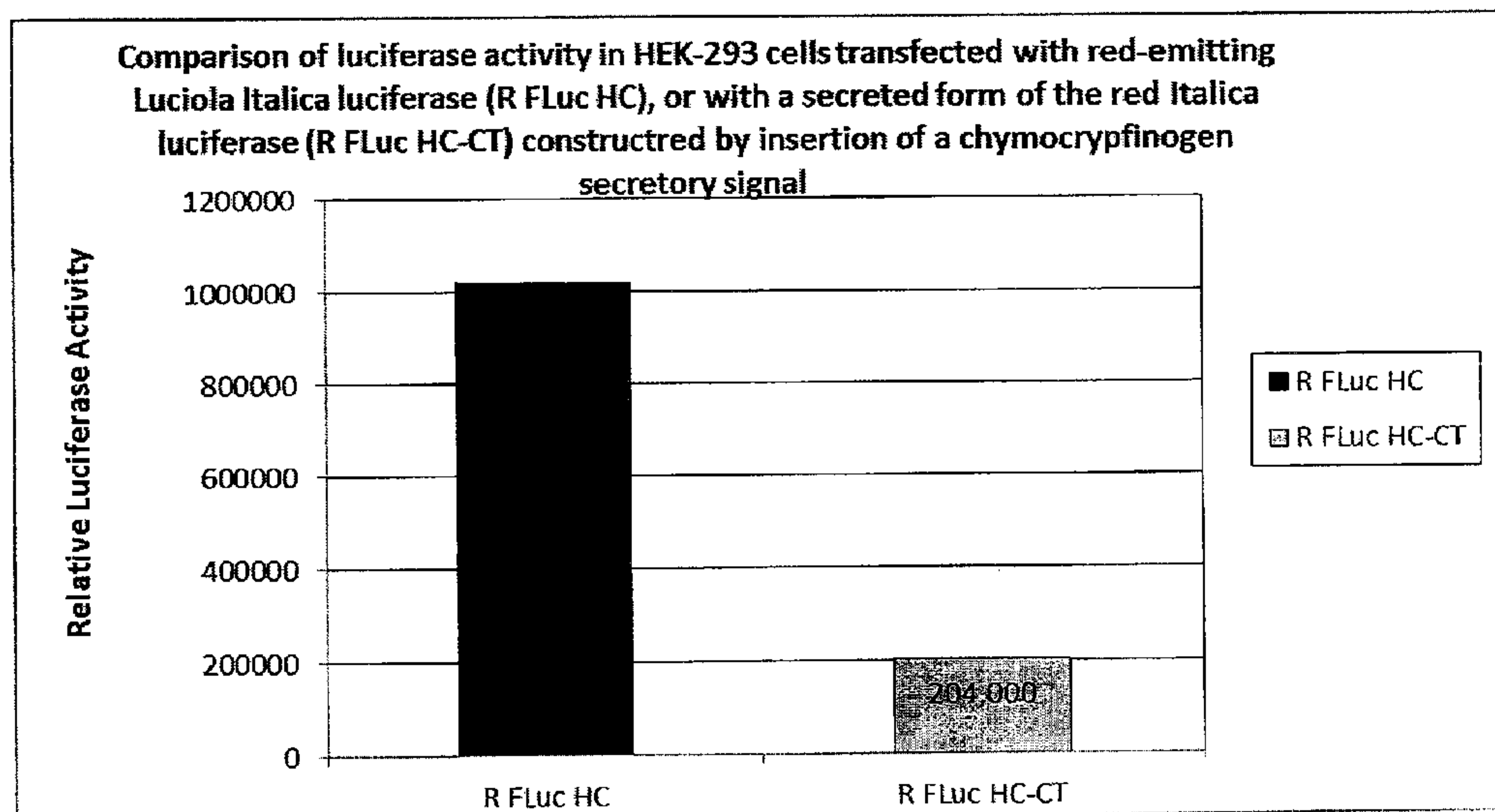


FIG. 11

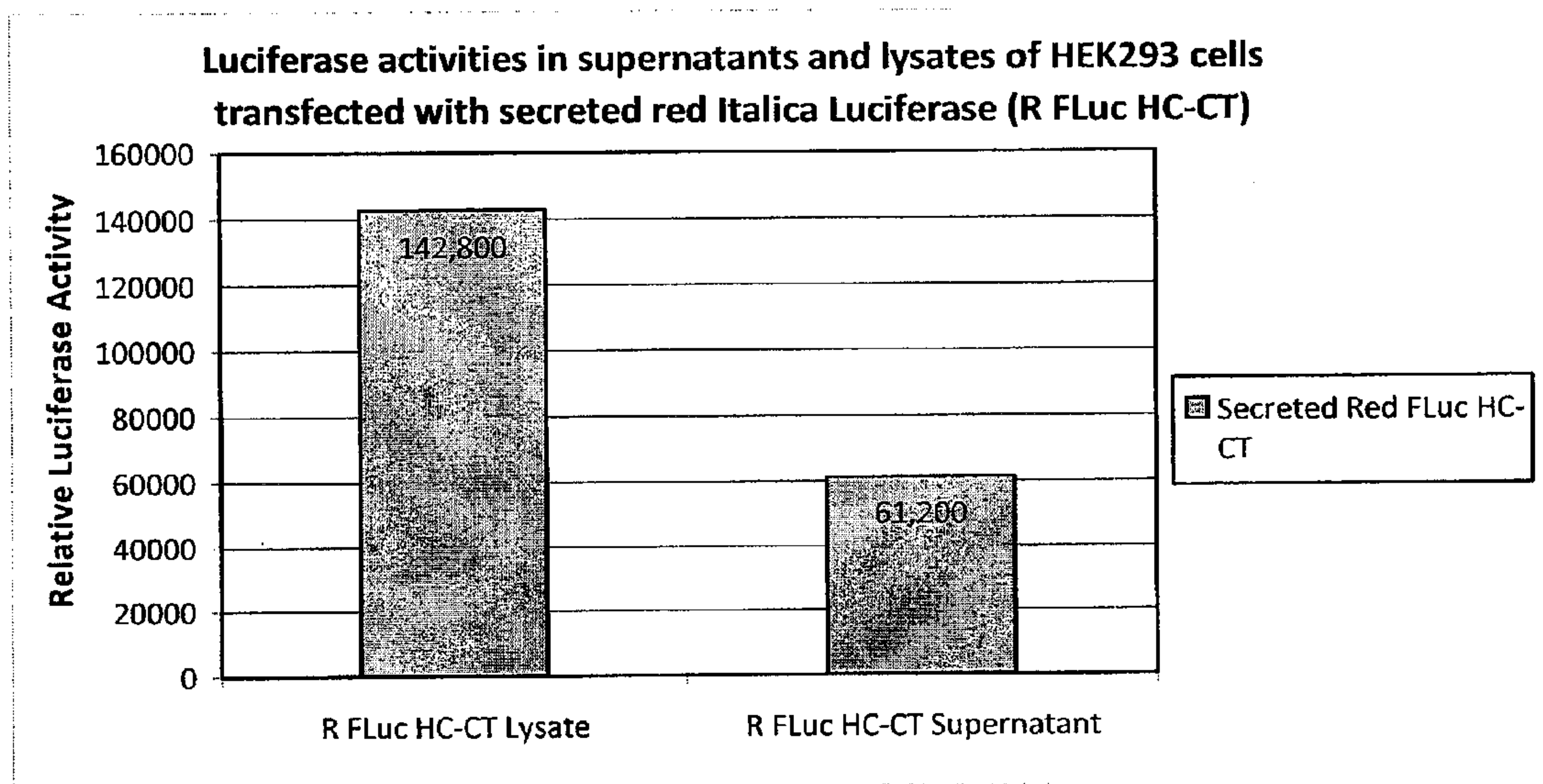


FIG. 12A

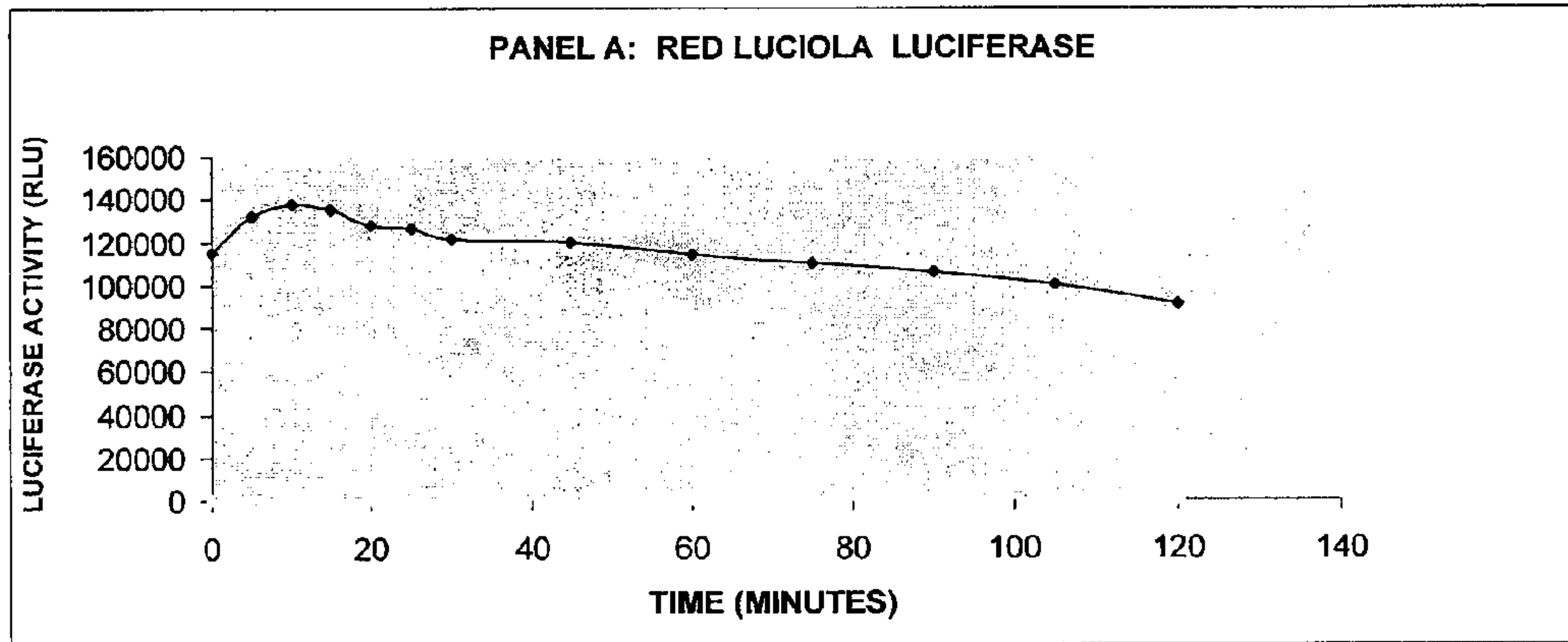


FIG. 12B

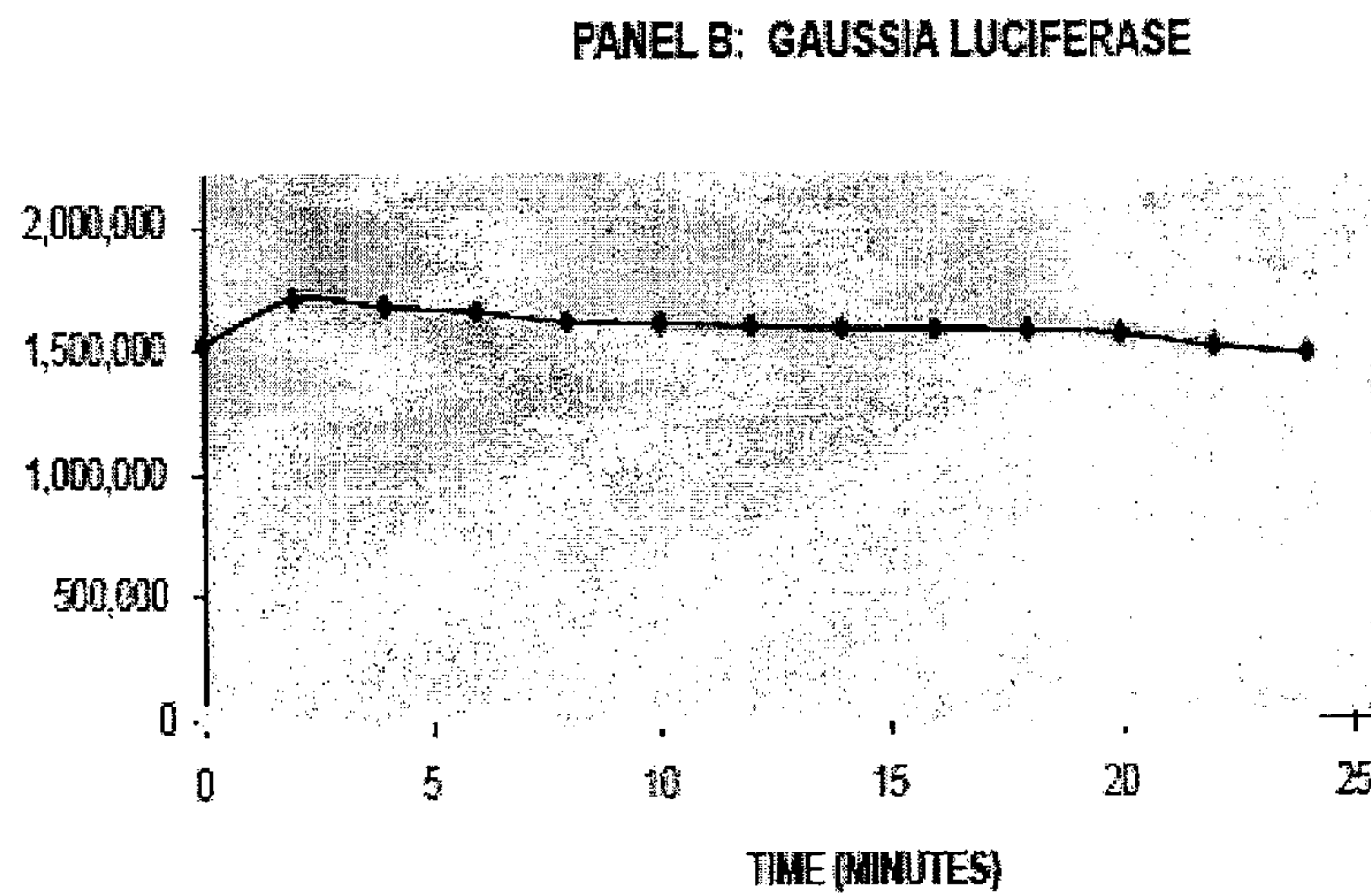


FIG. 12C

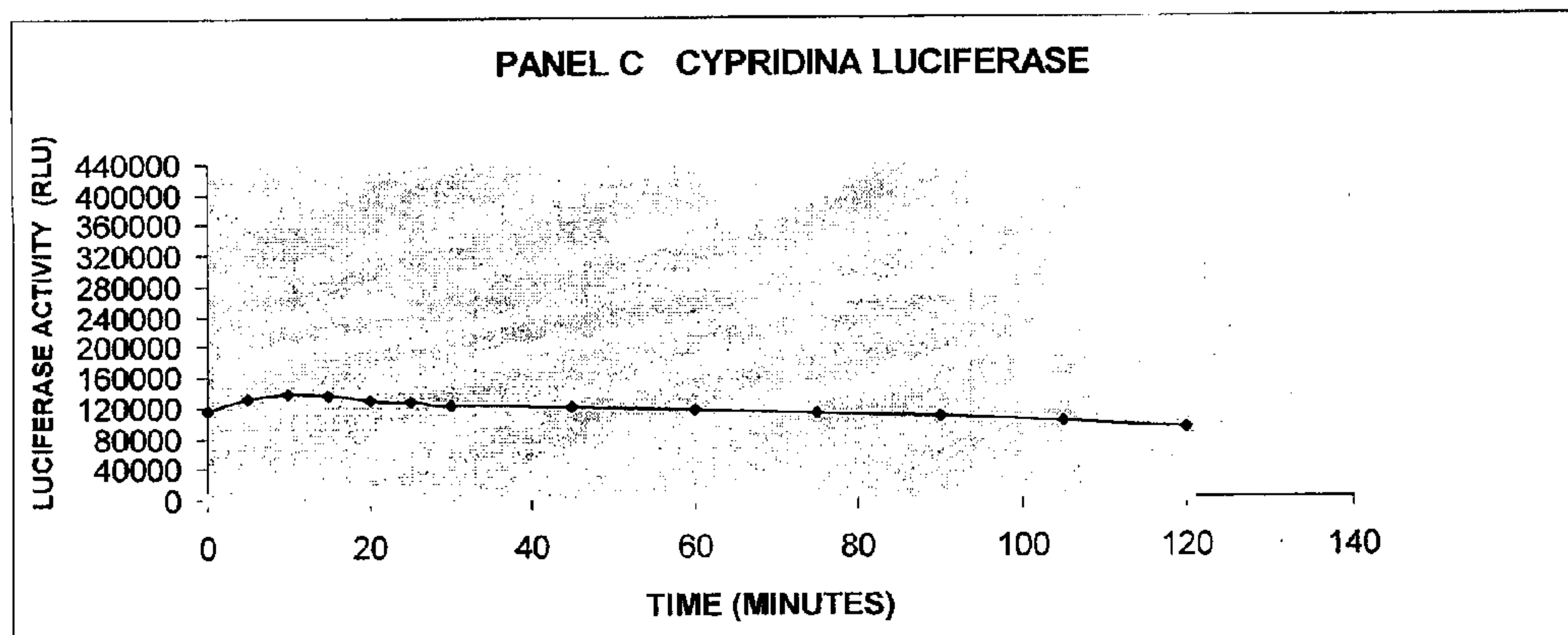


FIG. 12D

PANEL D: GREEN RENILLA LUCIFERASE

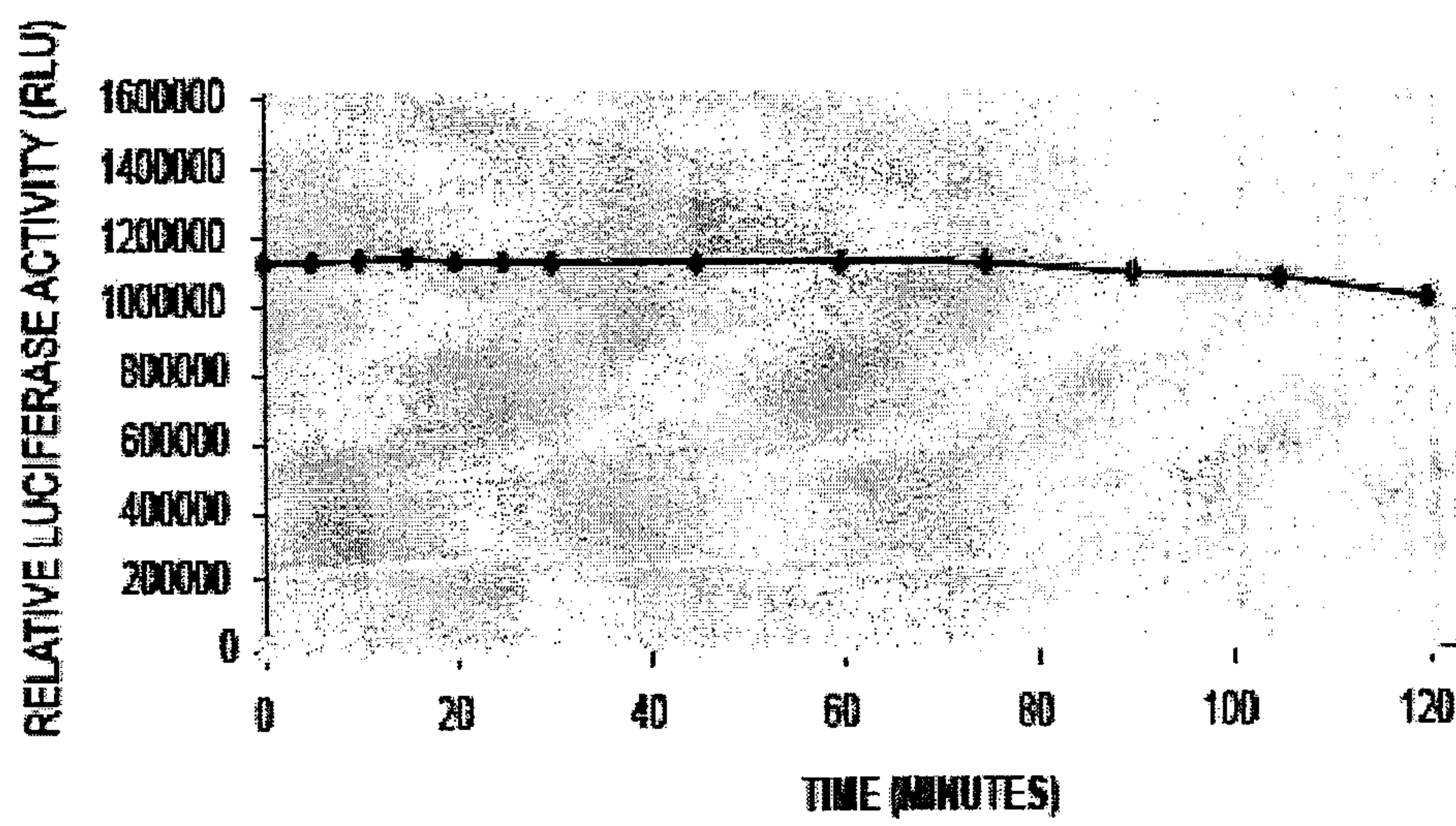


FIG. 13A

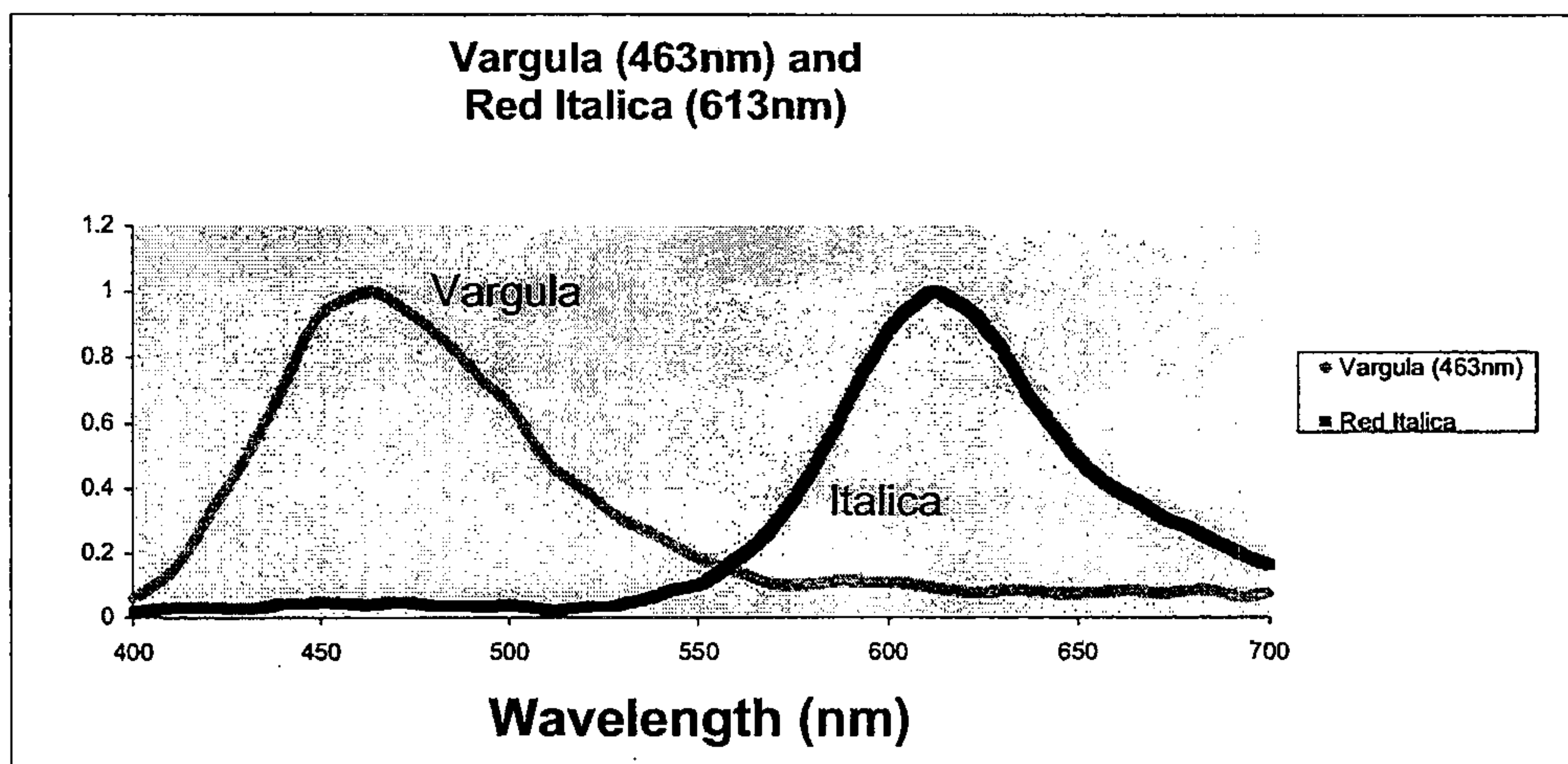


FIG. 13B

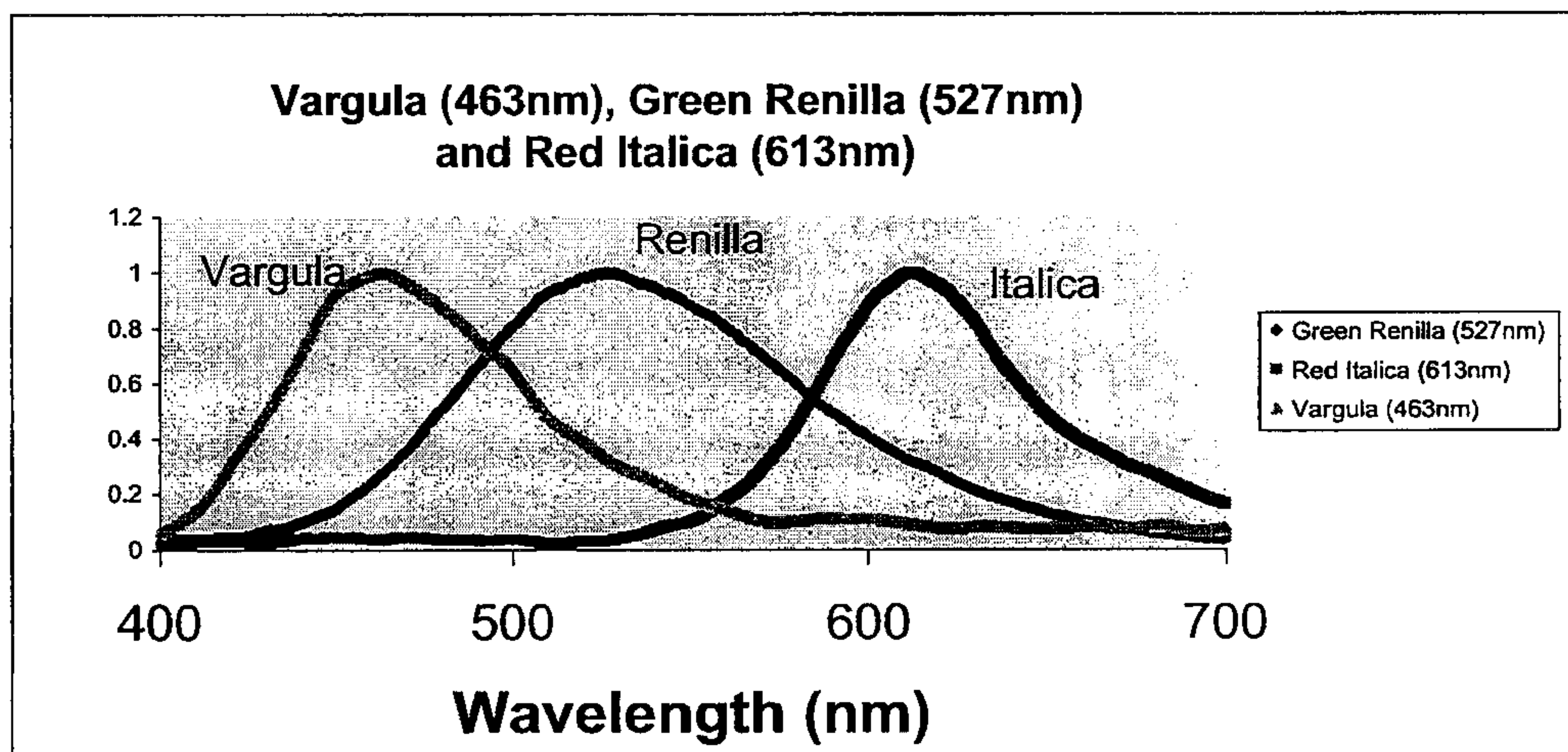


FIG. 14

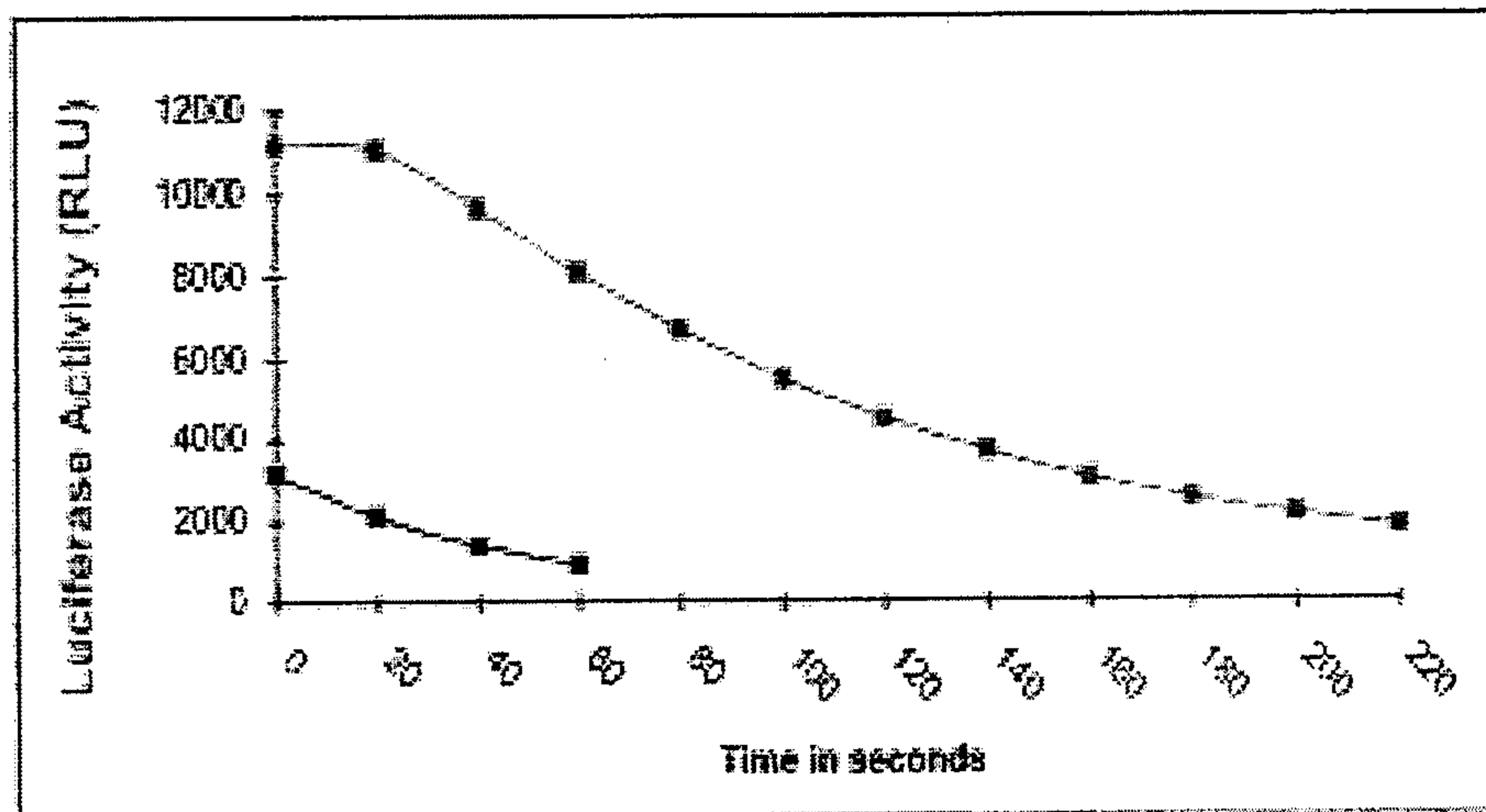


FIG. 15A

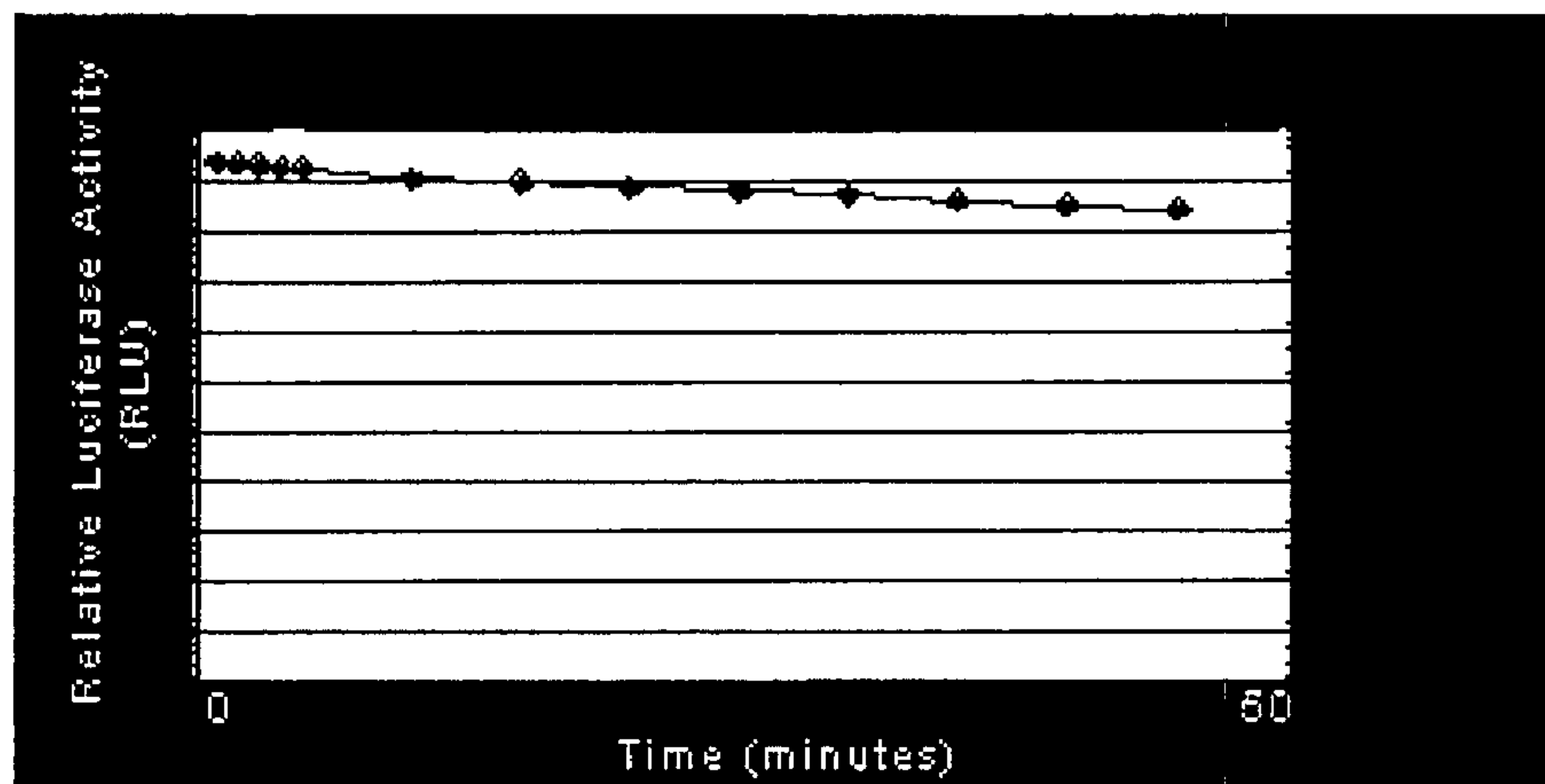


FIG. 15B

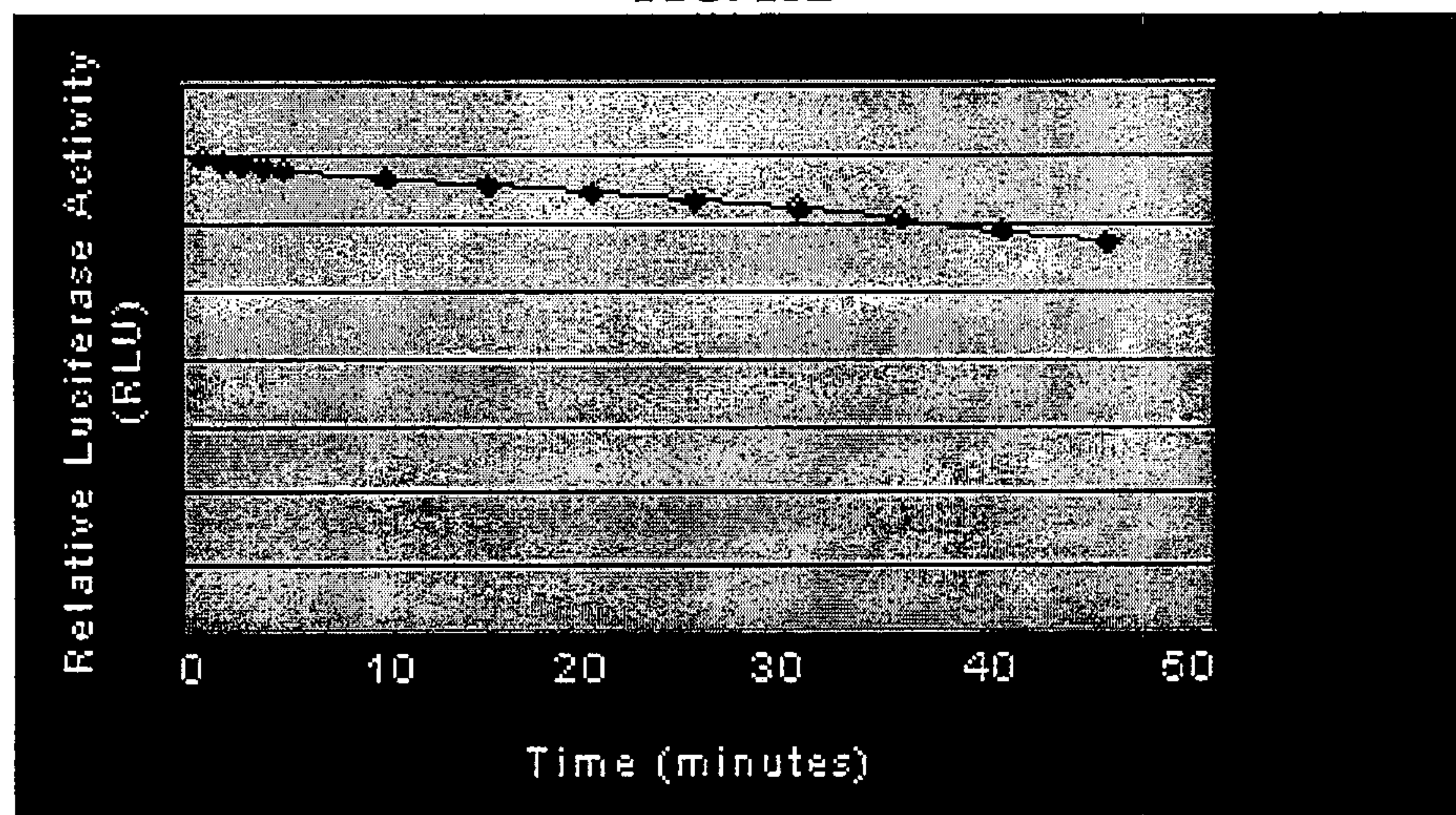


FIG. 16

Firefly Luciferase Assay reagent (FLAR-1)

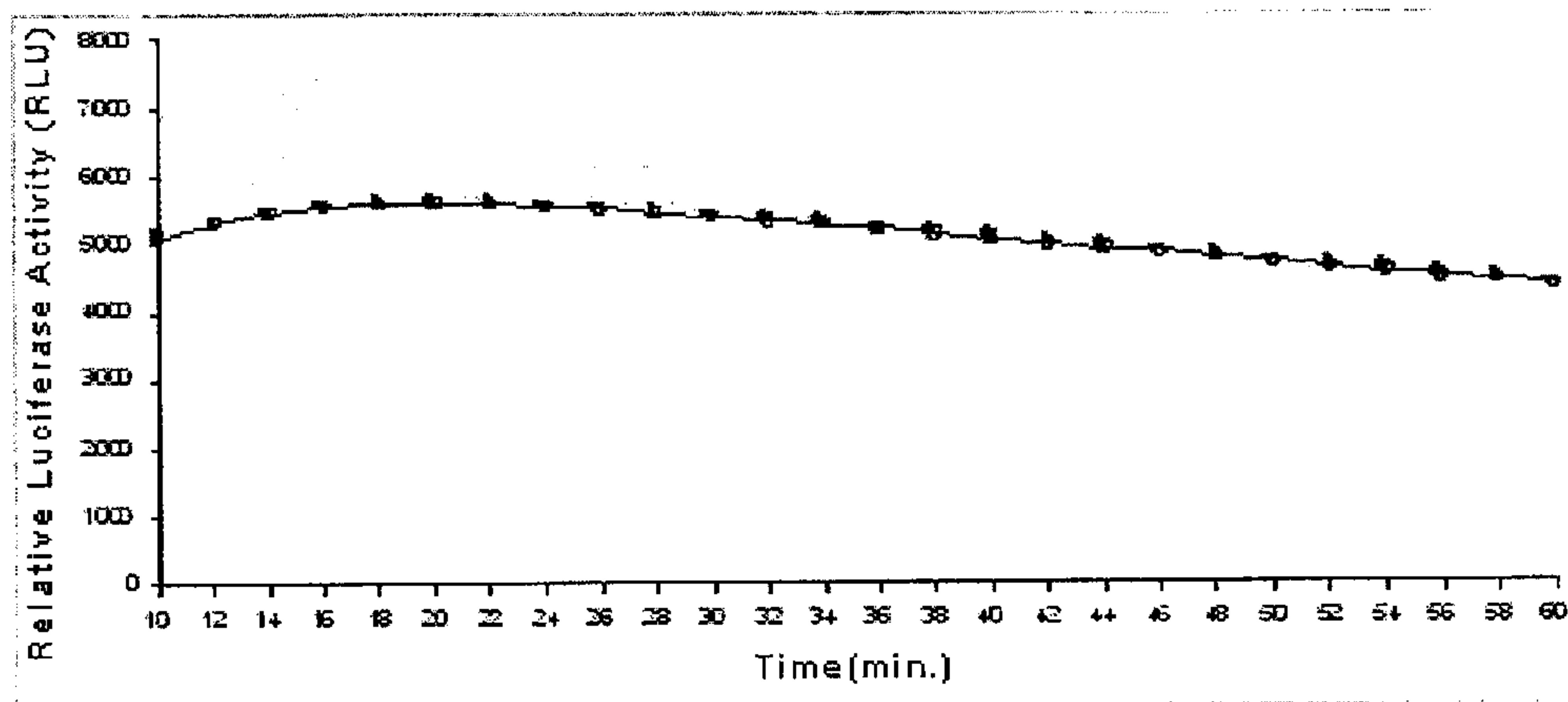


FIG. 17

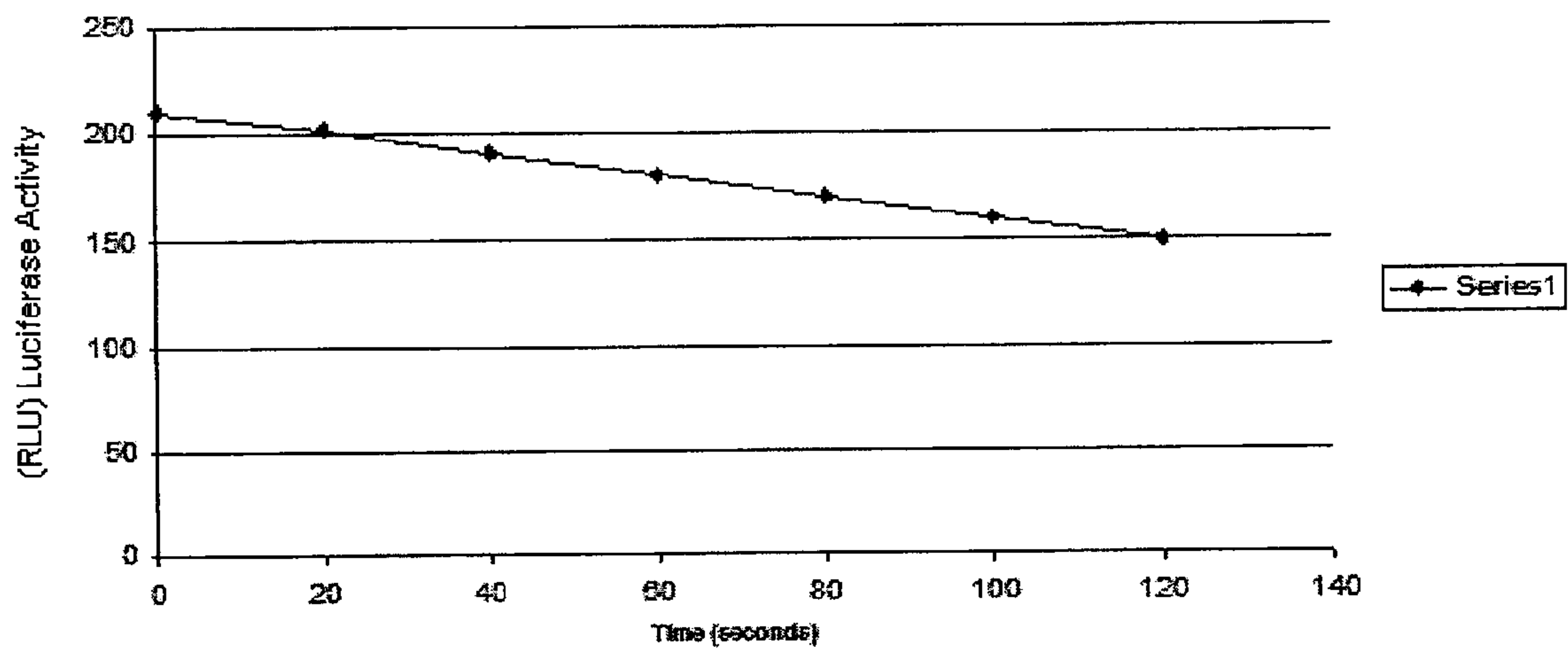


FIG. 18

Vargular Assay

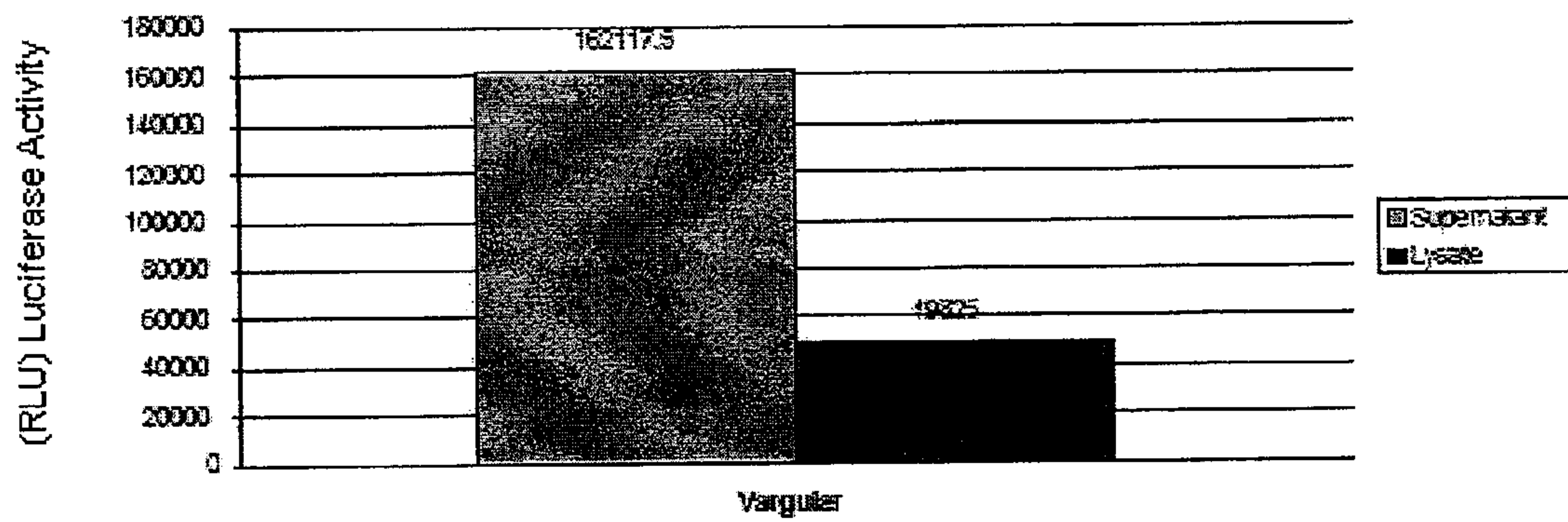


FIG. 19

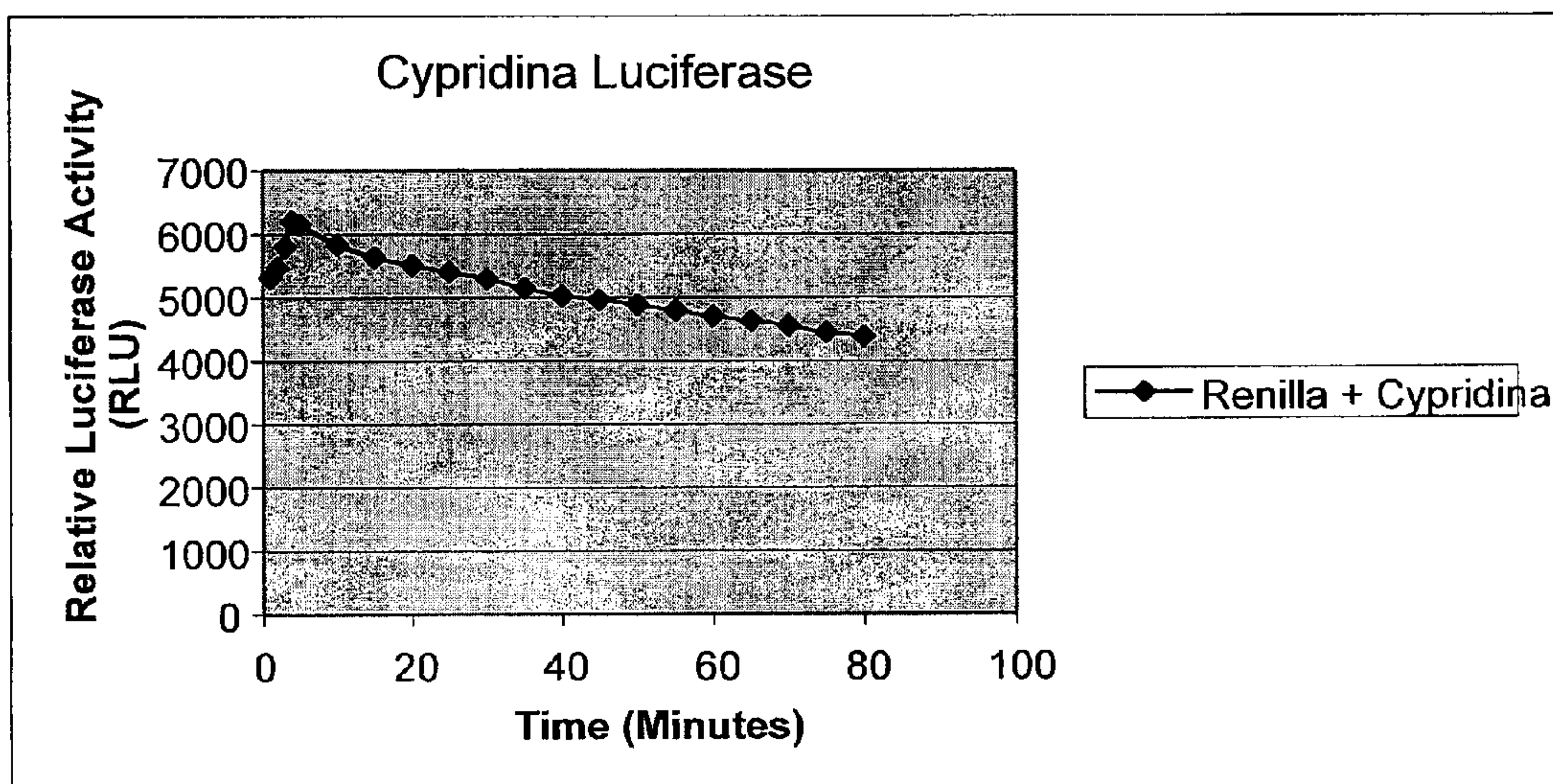


FIG. 20A

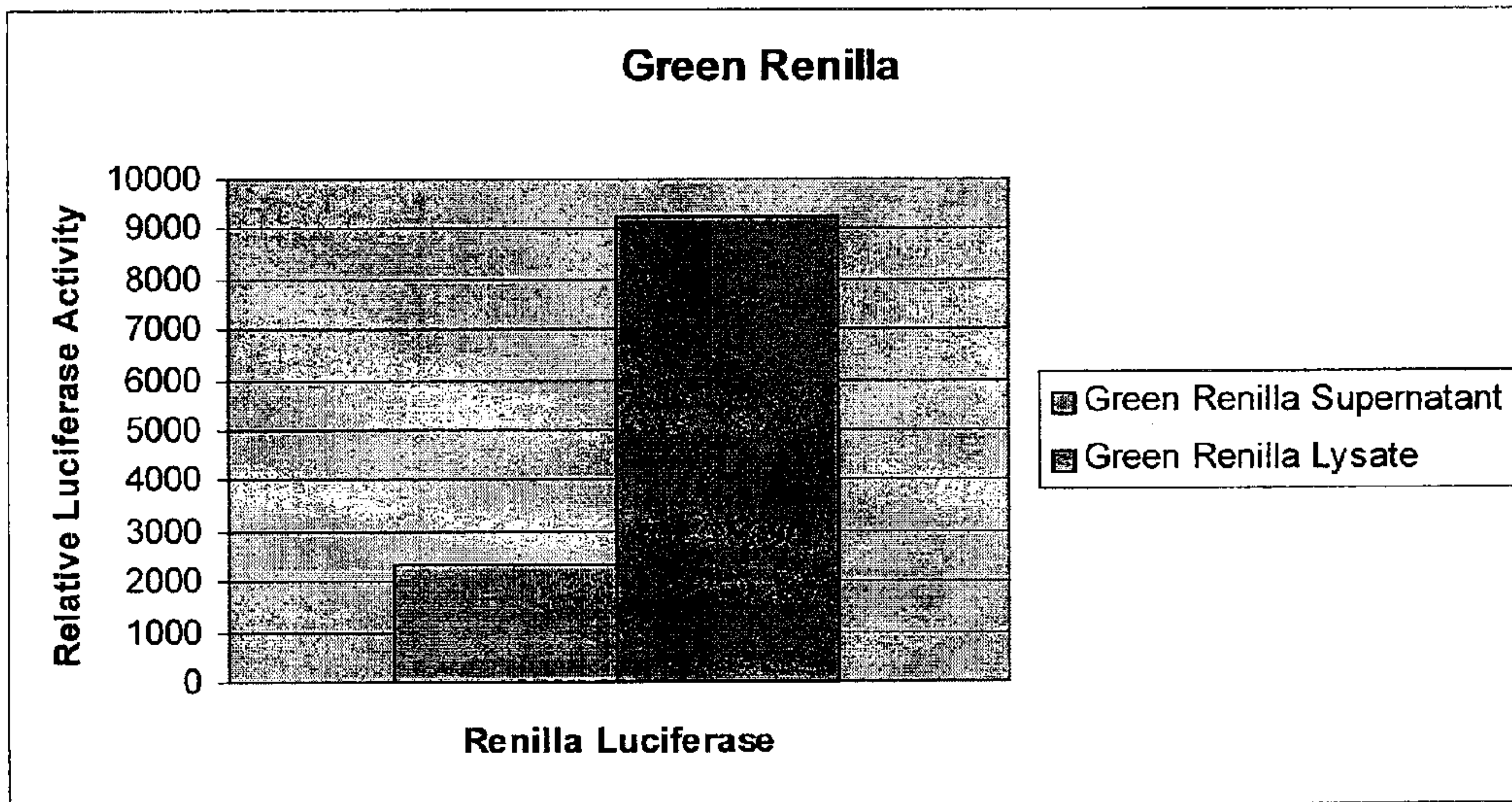


FIG. 20B

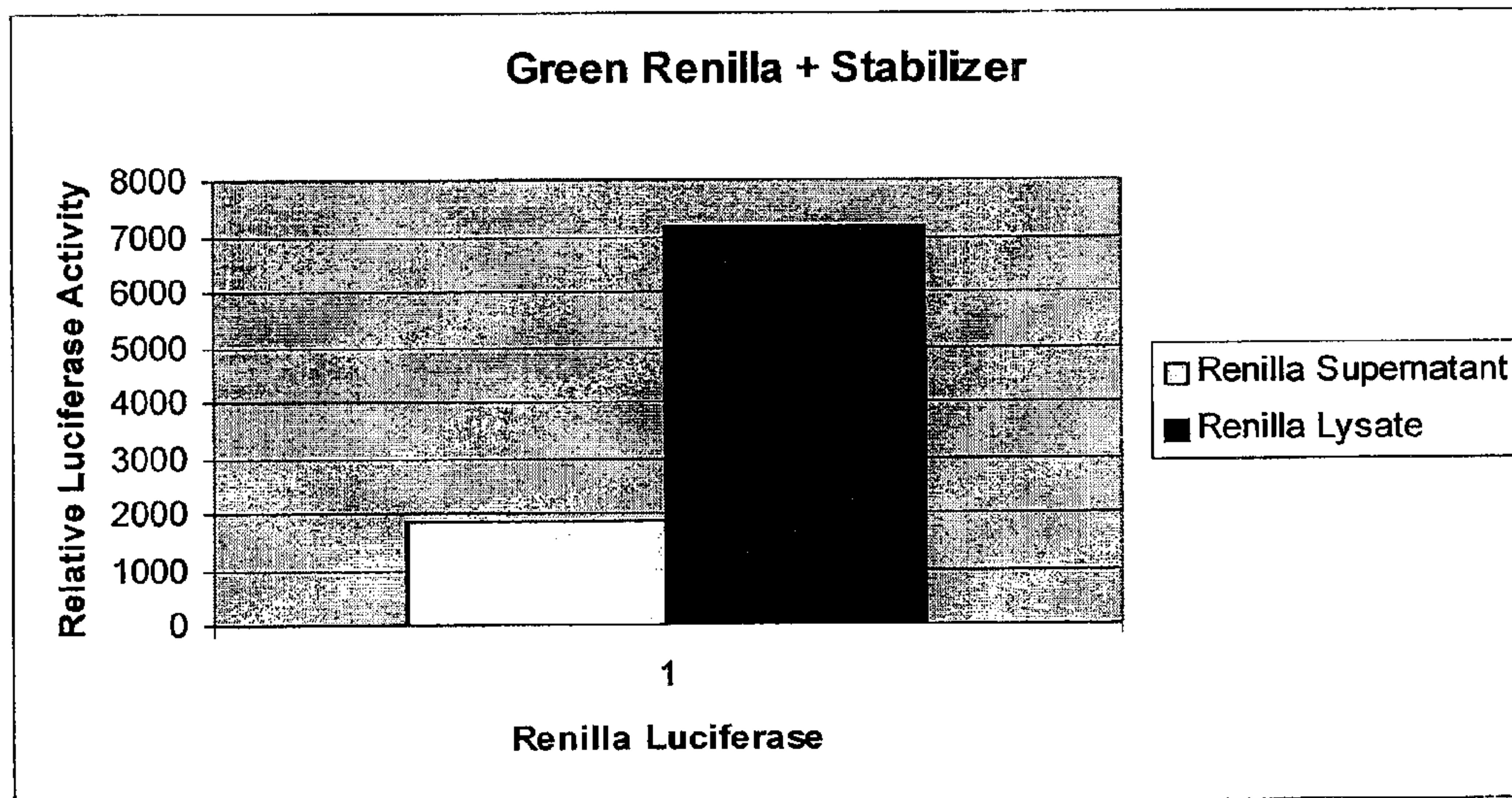


FIG. 21

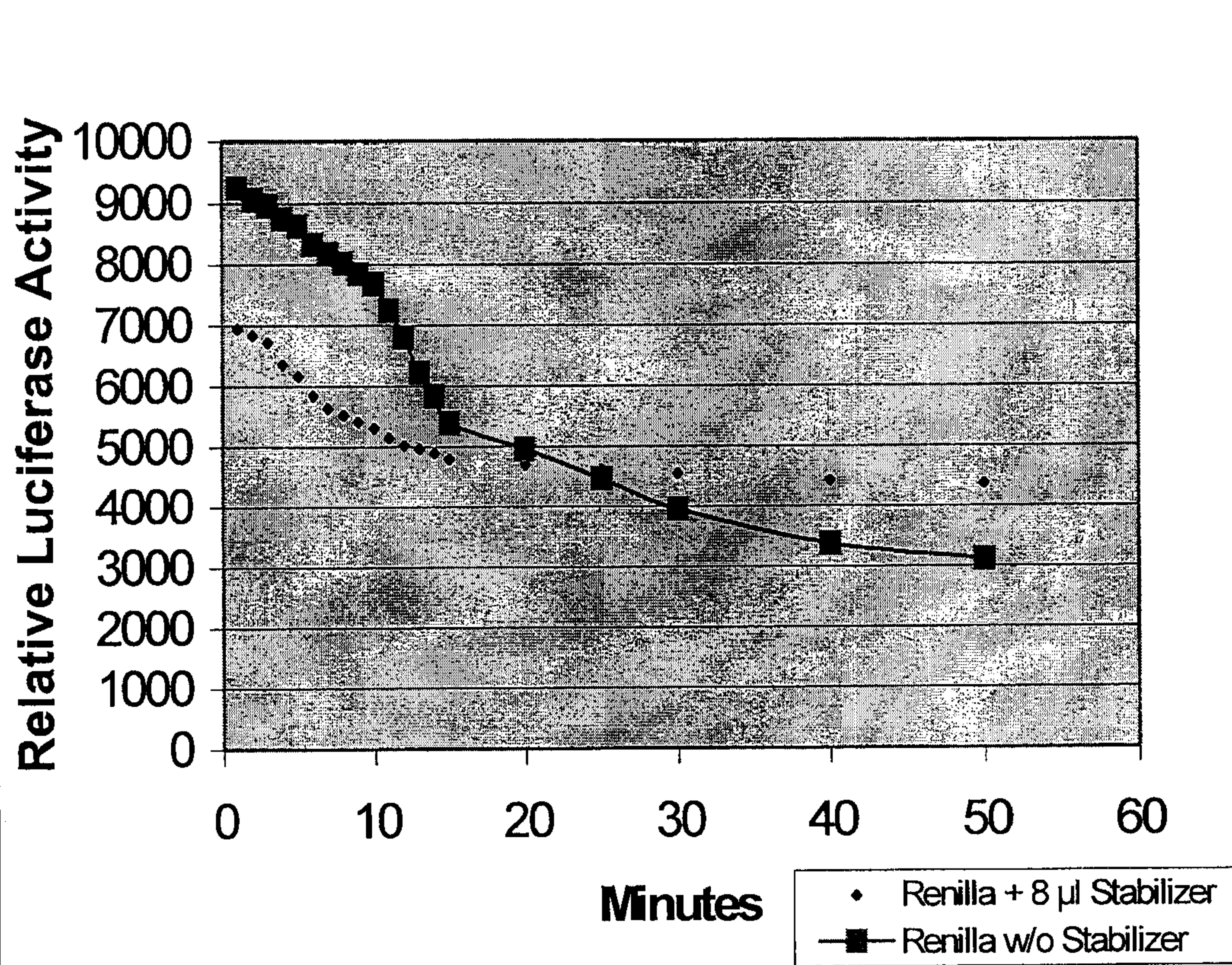


FIG. 22

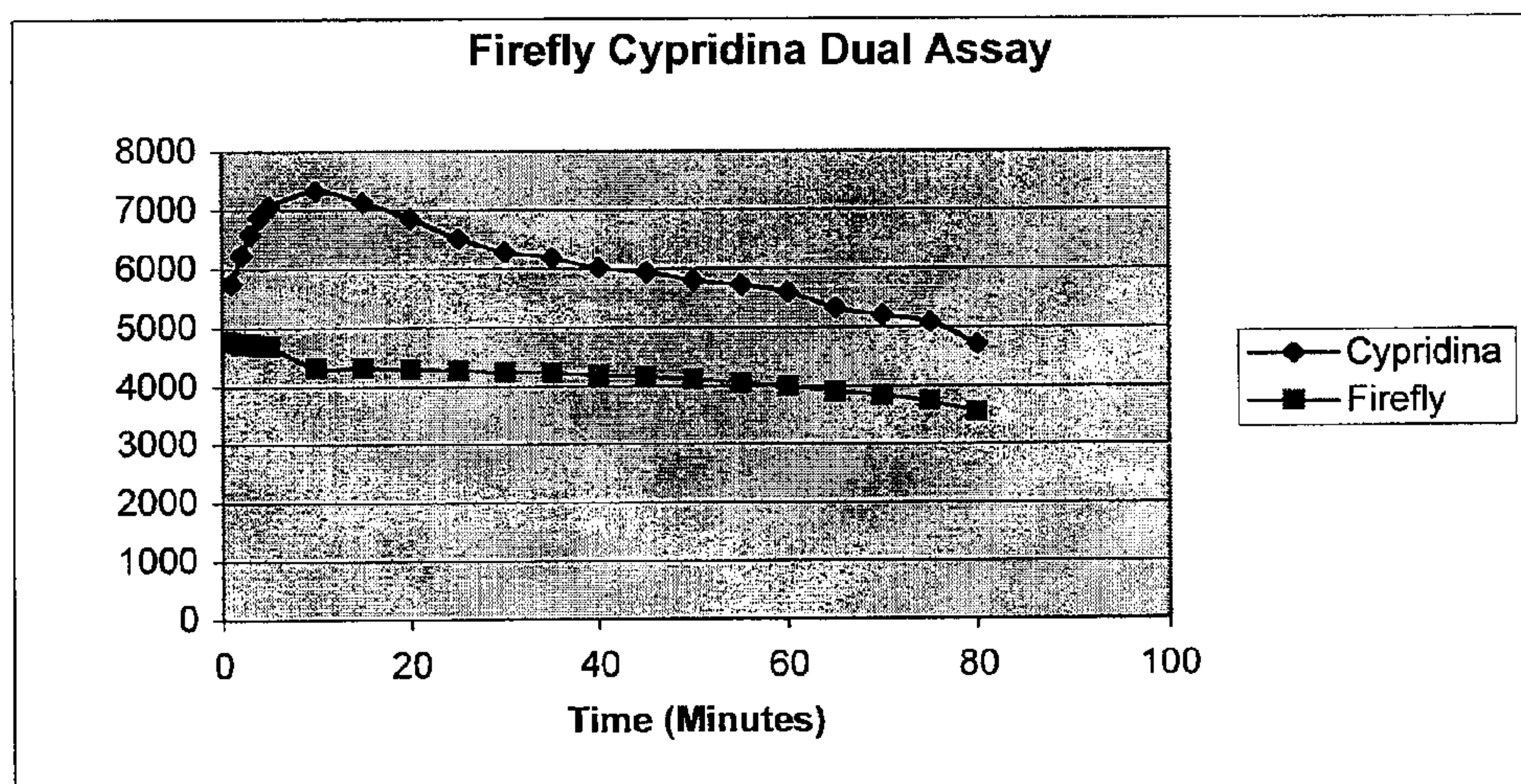


FIG. 23A

Dual Assay for Cypridina-Renilla Luciferase (DLAR-5)

Panel A

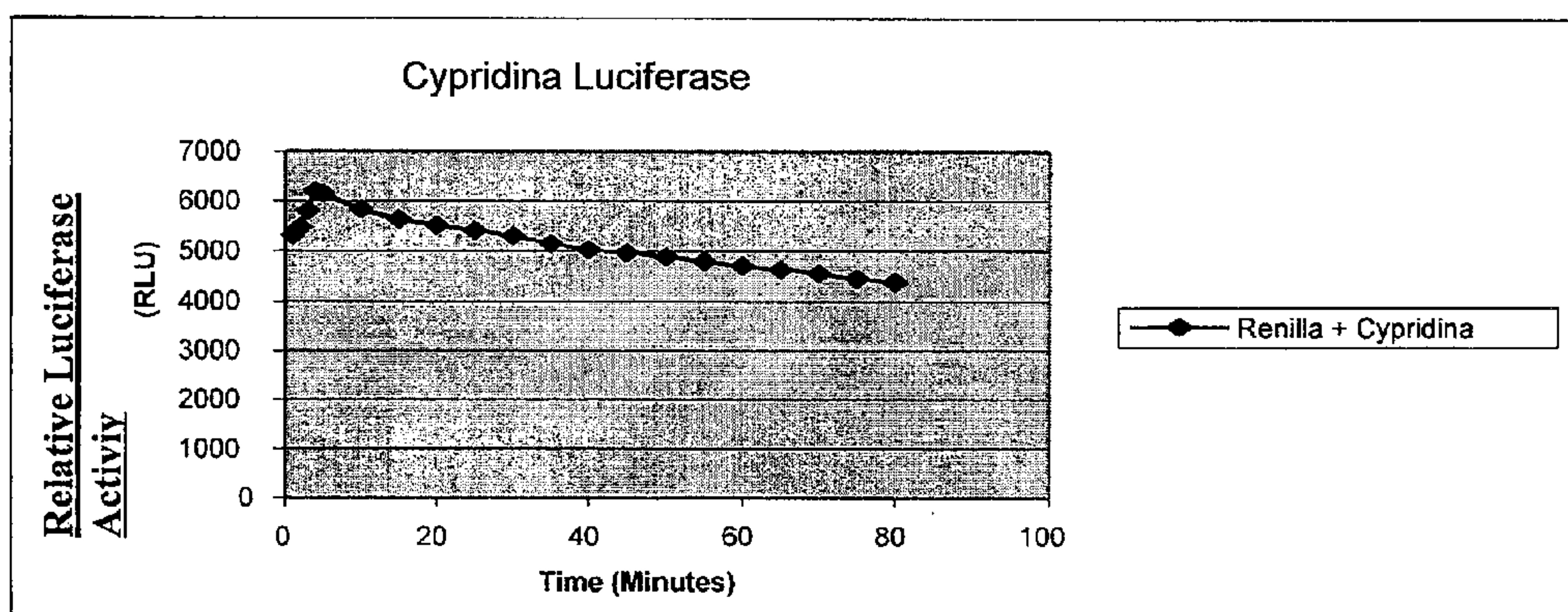


FIG. 23B

Panel B

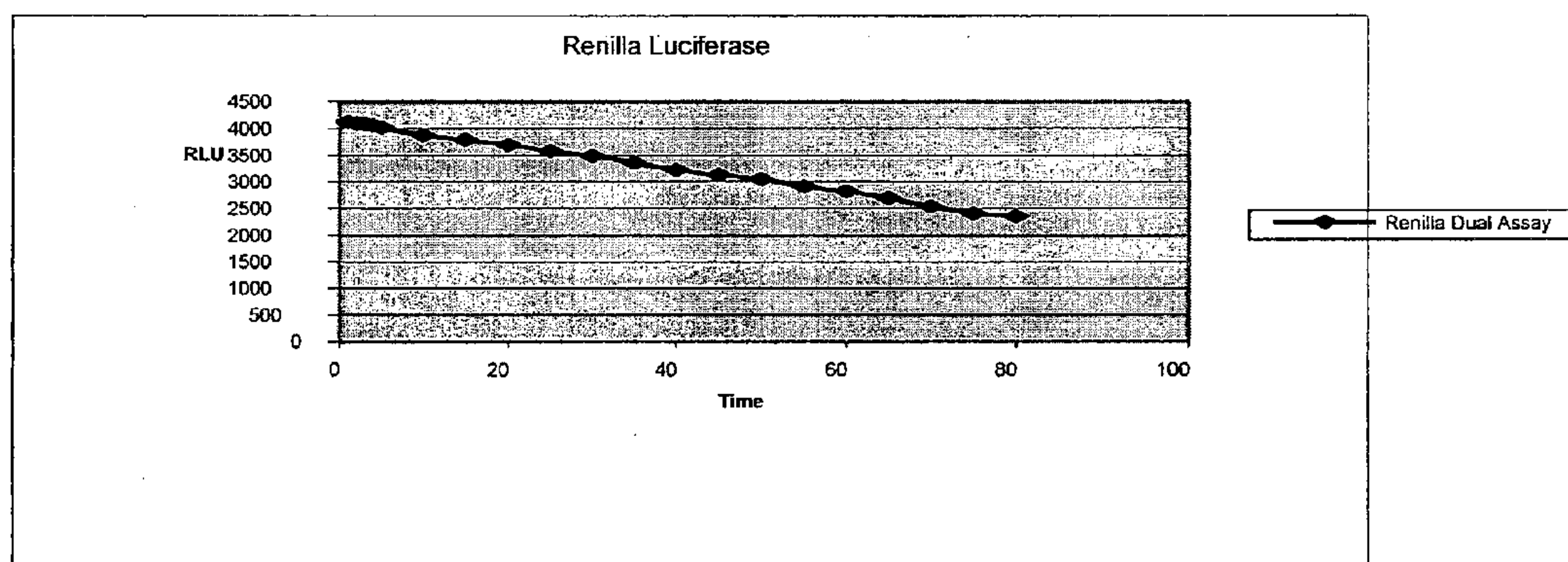


FIG. 24

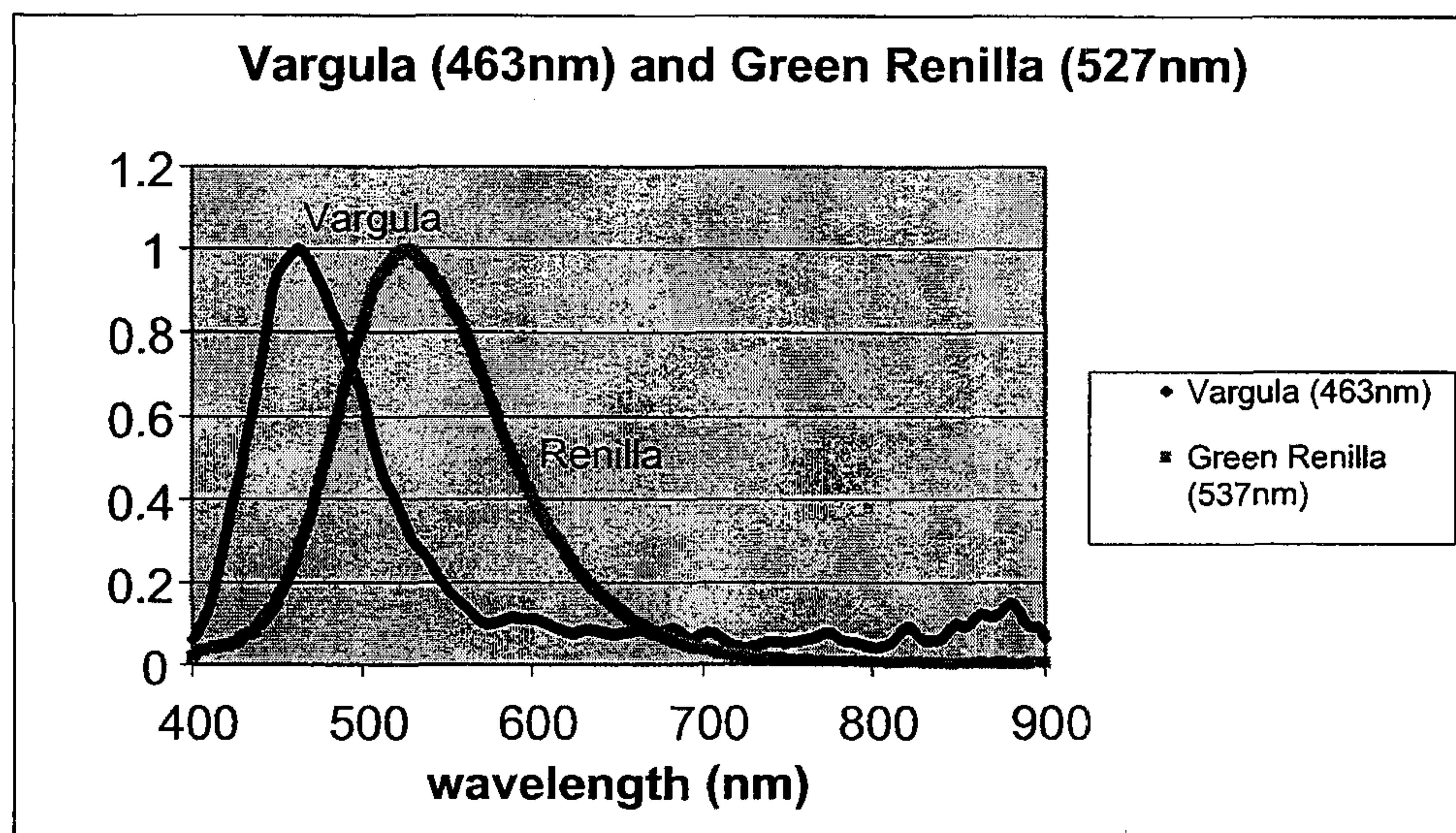


FIG. 25

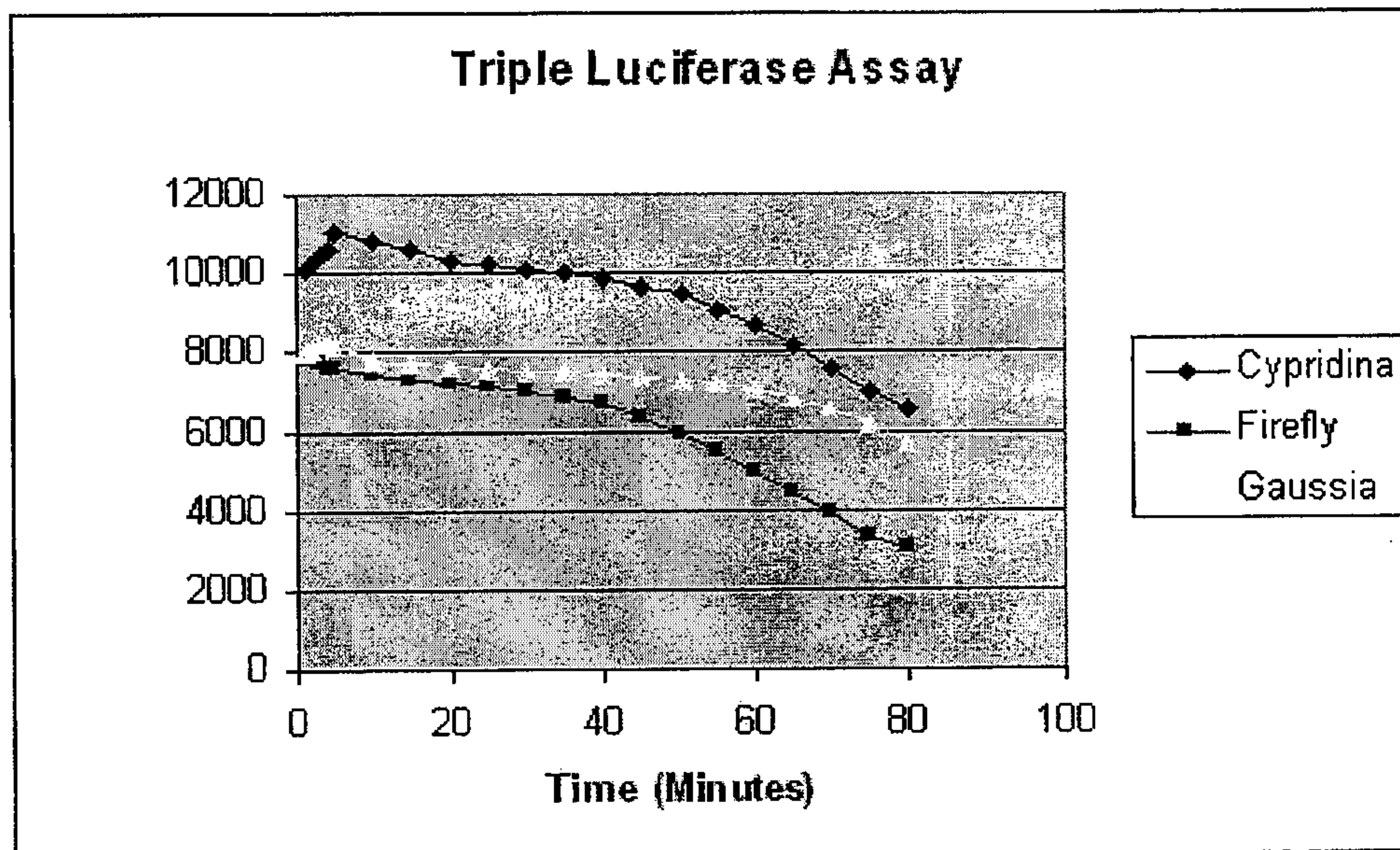


FIG. 26

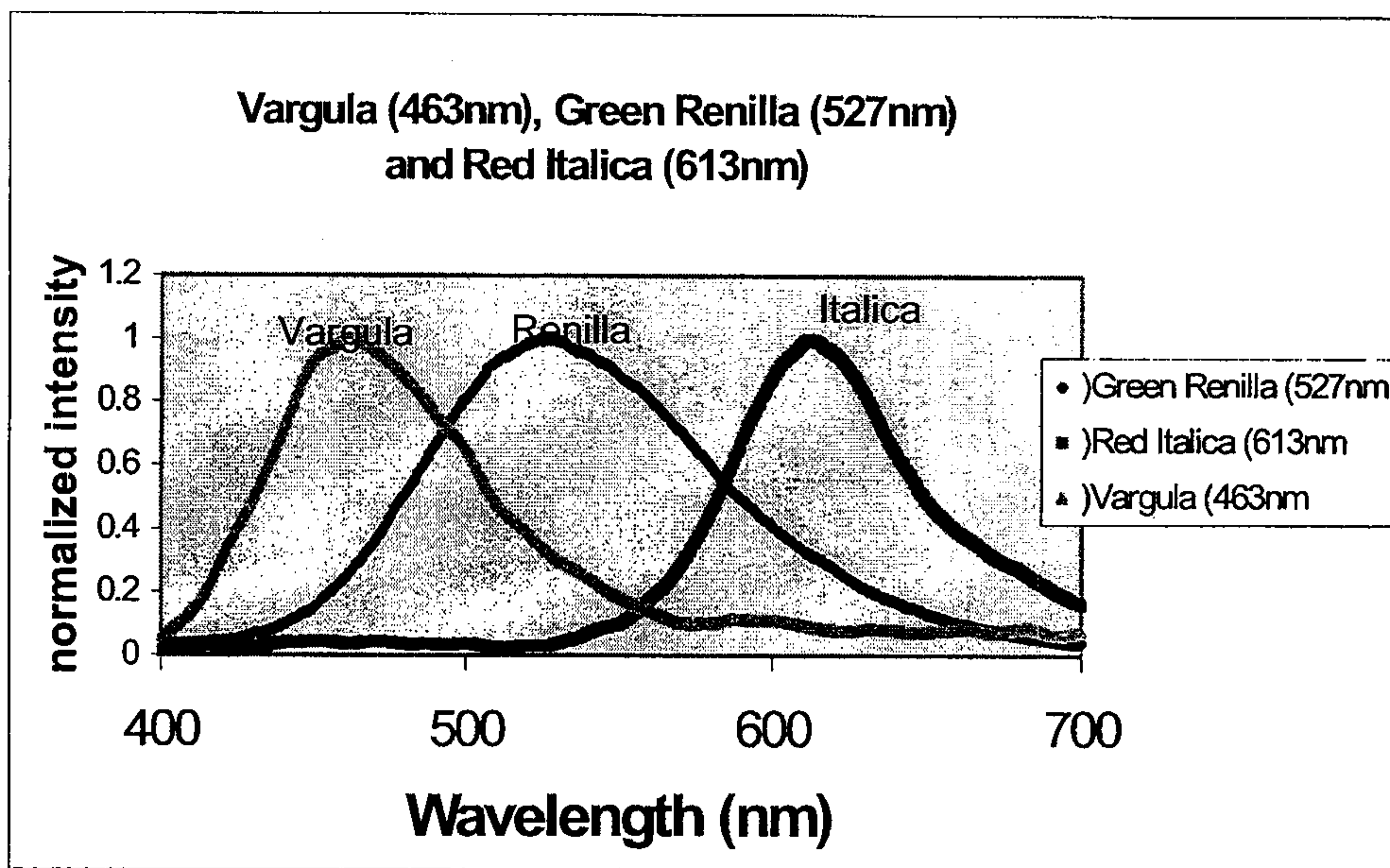


FIG. 27A

CMV Red Firefly Luciferase plasmid sequence:

ORIGIN

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggctcgt gagtagtgcg
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagttattaa tagtaatcaa ttacgggggc attagttcat agcccatata
301 tggagtccg cgttacataa ctacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgccatt gacgcaata atgacgtatg ttccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tattacggt aaactgcca ctggcagta catcaagtgt
481 atcatatgcc aagtacgccc cctatgacg tcaatgacgg taaatggccc gcctggcatt
541 atgccagta catgacctta tgggacttc ctactggca gtacatctac gtattagta
601 tcgctattac catggtgatg cggtttggc agtacaatca tgggctgga tagcggttg
661 acicacgggg attccaagt ctccaccca ttgacgtcaa tgggagttg tttggcacc
721 aaaatcaacg ggactttcca aatgtcgtg acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841 ctgctactg gctatcgaa attaatcga ctactatag ggagaccaa gcttggacc
901 gagctcggat ccagccacca tggaaacaga aagagaagaa aacgttgtct acggcccact
961 gccattctac ccgatcgagg agggctctgc cggcatcaa tgcacaagt acatgcaaca
1021 atacgccaag ctggcgcca tcgctctcag taacgcctg acaggcgtcg acatcagcta
1081 ccagcagtac ttgacatca cgtgcagact cgccgaggct atgaagaact acggcatgaa
1141 gccagaagga cacatcgtc tctgtagcga gaactgcgaa gagttctca ttctgttct
1201 ggctggtctt tacatcggag ttacagtcgc gccaactaac gaaattata cacttagaga
1261 gctgaaccac agtctgggga tagccaacc tactatcgt tctctagca ggaagggcct
1321 gcccaaagtg ctgagggtc agaagaccgt gacttgcac aaaaccattg tcatcctgga
1381 cagtaaggtc aactcggcg gttatgactg cgtagagacc ttcattaaga aacacgtcga
1441 gctgggctt cctgccacct catttggcc catcgacgtc aaagaccgga agcaccacat
1501 tgctctgctt atgaactct cgggtccac agggctgccc aaaggagtag agatcactca
1561 cgaggccctg gtcacgagat tctctcagc taaggaccct atatacggca atcaggtggc
1621 cccaggtagc gctatcctga ctgctgtgcc ttccaccac ggctcggaa tgttactac
1681 tttgggtac ttgctcgc gttaccgat tgcattgct actaagttcg acgaggagct
1741 ttctcgcg acactcagg attacaagt cactacagta atctgggtc cgacactgtt
1801 cgcaattctt aataggctg agctcctga taagttgac ctcttaacc tgactgaaat
1861 agccagcgtt ggtgctccac ttgccaagga gatcggcgag gctgtgcaa gaagattcaa
1921 cctcccaggc gtccggcagg gatatggact caccgagact accagtgcct ttatcatcac
1981 tctaagggc gacgacaagc cgggagccag cggcaaggtc gtgcctctgt tcaaggtgaa
2041 gattattgac ctcgatacca agaaaacgtt ggggtgcaac agacggggag aaatctcgt
2101 gaaaggacca tctctatgt tgggatacac gaacaatcct gaagccacca gagaaactat
2161 tgacgaggaa ggctggctgc acacgggtga catcgggtac tacgacgagg atgagcactt
2221 cttatagtc gaccgcctga aatctctcat taagtataaa ggataccaag tgccaccagc
2281 tgaactggag tctgtgctc tgcaacacc taacattaga gatgctggg tggccgggg
2341 tccgacagc gaggcaggcg agctgcctgg agccgtcgtt gtgatggaaa agggaaagac
2401 aatgactgag aaagaaatc tagactatgt aaactcccag gtggtaacc acaagcggct
2461 gaggggccc gtgcggctc tagatgaagt cccaagggg ctacacaggaa agatcgacgc
2521 gaaagtatc agggagatac tcaagaaacc tcaagcaggt gggtagtcta gaaataattc
2581 ttactgtcat gccaagtaag atgctttct gtgtgcaat agcaggcatg ctggggatgc
2641 ggtgggctct atggctctg aggcgaaag aaccagctgg ggctctaggg ggtatccca
2701 cgcgccctgt agcggcgc at taagcgcggc ggggtgggtg gttacgcgca gcgtgaccgc
2761 tacactgcc agcgcctag cgcgcctcc ttgccttc ttccctct tctcgcac
2821 gttcggcggc ttccccgc aagctctaaa tggggcatc ccttaggg tccgattag
2881 tgcttacgg cacctcgacc ccaaaaaact tgattaggg gatggtcac gtatggggc
2941 atgcctga tagacgggtt ttgccctt gacgtggag tccacgtct ttaatagtg
3001 actctgtc caaactggaa caaactcaa ccctatctg gtctattct ttgattata
3061 agggatttg gggattcgg cctatgggt aaaaaatgag ctgattaac aaaaattaa

FIG. 27B

3121 cgcgaaattaa ttctgtggaa tgtgtgtcag ttaggggtgtg gaaagtcccc aggctcccca
3181 ggcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccagggt gtggaaagtc
3241 cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt cagcaacccat
3301 agtccccgcc ctaactccgc ccatccccgc cctaactccg cccagttccg cccattctcc
3361 gccccatggc tgactaattt ttttattta tgcagaggcc gaggccgcct ctgcctctga
3421 gctattccag aagtagtgag gaggctttt tggaggccta ggcttttga aaaagctccc
3481 gggagctgt ataccattt tcggatctga tcaagagaca ggatgaggat cgtttcgcat
3541 gattgaacaa gatggattgc acgcaggctc tccggccgct tgggtggaga ggctattcgg
3601 ctatgactgg gcacaacaga caatcggctg ctctgatgcc gccgtgttcc ggctgtcagc
3661 gcagggggcg ccggtcttt ttgtcaagac cgacctgtcc ggtgccctga atgaactgca
3721 ggacgaggca gcgcggtat cgtggctggc cacgacgggc gttccttgcg cagctgtgct
3781 cgacgtgtc actgaagcgg gaagggactg gctgctattg ggcaagtgc cggggcagga
3841 tctctgtca tctcacctg ctctgccga gaaagtatcc atcatggctg atgcaatgcg
3901 gcggctgcat acgctgtatc cggctacctg cccattcgac caccaagcga aacatcgcat
3961 cgagcgagca cgtactcggg tggagccgg tcttctgat caggatgatc tggacgaaga
4021 gcatcagggg ctgcgcccag ccgaactgtt cgccaggctc aaggcgcgca tgcccagcgg
4081 cgaggatctc gtcgtgacc atggcgatgc ctgctgccg aatatcatgg tggaaaatgg
4141 ccgctttct ggattcatc actgtggccg gctgggtgtg gcggaccgct atcaggacat
4201 agcgttggct acccgtgata ttgctgaaga gcttggcggc gaatgggctg accgcttct
4261 cgtgctttac ggtatcgccg ctcccgattc gcagcgcac gccttctac gccttctga
4321 cgagttctc tgagcgggac tctggggtc gaaatgaccg accaagcgcg gcccaacctg
4381 ccatcacgag atttcgattc caccgcccgc ttctatgaaa ggttgggctt cggaatcgtt
4441 ttccgggacg ccggtggat gatcctccag cgcggggatc tcatgctgga gttctcggc
4501 caccccaact tgttattgc agcttataat gttacaaat aaagcaatag catcacaat
4561 ttcacaaata aagcatttt tcaactgcat tctagtgtg gttgtccaa actcatcaat
4621 gtatctatc atgtctgat accgtcgacc tctagctaga gcttggcgta atcatggtca
4681 tagctgttc ctgttgaaa ttgtatccg ctcaaatc cacacaacat acgagccgga
4741 agcataaagt gtaaagcctg ggggtcctaa tgagttagct aactcacatt aattgcgtg
4801 cgctcactgc ccgcttcca gtcgggaaac ctgtcgtgcc agctgcatta atgaatcggc
4861 caacgcgagg ggagaggcgg ttgcgtatt gggcgtctt ccgcttctc gctcactgac
4921 tcgctgctc cgtcgttcg gctgcggcga gcggtatcag ctactcaaa ggccgtaata
4981 cggttatcca cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa
5041 aaggccagga accgtaaaaa ggccgcgtg ctggcgttt tccataggct ccgccccct
5101 gacgagcatc acaaaaatcg acgctcaagt cagagggtgc gaaaccgcg accactataa
5161 agataccagg cgtttcccc tggagctcc ctctgctgct ctctgttcc gaccctgccg
5221 cttaccgat accgtccgc ctttctctt tcgggaagcg tggcgtttc tcaatgctca
5281 cgctgtagg atctcagttc ggttaggtc gttcgtcca agctgggctg tgtgcacgaa
5341 cccccgtc agcccgaccg ctgcgctta tccgtaact atcgtcttga gtccaaccg
5401 gtaagacacg acttatgcc actggcagca gccactggtg acaggattag cagagcagg
5461 tatgtaggc gtgctacaga gttctgaag tggggccta actacggcta cactagaagg
5521 acagtattg gtatctgctc tctgctgaag ccagttacct tcggaaaaag agttggtagc
5581 tctgatccg gcaaaaaac caccgctgtt agcgggtgtt tttgtttg caagcagcag
5641 attacgcgca gaaaaaagg atcaagaa gatccttga tctttctac ggggtctgac
5701 gctcagtga acgaaaactc acgttaaggg atttgttca tgagattac aaaaaggatc
5761 ttcacctaga tcttttaa taaaaatga agttttaa caatctaaag tataatgag
5821 taaacttgt ctgacagta ccaatgctta atcagttagg cacctatctc agcgatctg
5881 ctattctgt catccatagt tgcctgactc cccgtcgtt agataactac gatcgggag
5941 ggcttaccat ctggcccag tctgcaatg ataccgcgag acccagctc accggctcca
6001 gattatcag caataacca gccagccgga agggccgagc gcagaagtgg tctgcaact
6061 ttatccgct ccatccagtc tattaatgt tgcgggaag ctagagtaag tagttcgcca
6121 gftaatagt tgcgcaactg tttgctt gctacaggca tctgtgtc acgctcgtc
6181 tttggtatg ctctatcag ctccggttcc caacgatcaa ggcgagttac atgatcccc
6241 atgtgtgca aaaaagcgt tagctctc ggtctccga tctgtcag aagtaagtg
6301 gccgcagtgt taccatcat ggttatggca gcaactgata attctctac tctcatgcca

FIG. 27C

6361 tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt
6421 atgcggcgac cgagttgctc ttgccggcg tcaatacggg ataataccgc gccacatagc
6481 agaactftaa aagtgctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc
6541 ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg atcttcagca
6601 tctttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa
6661 aagggataa gggcgacacg gaaatgtga atactcatac tcttccttt tcaatattat
6721 tgaagcattt atcagggfta ttgtctcatg agcggataca tatttgaatg tatttagaaa
6781 aataaacaaa taggggtcc gcgcacattt ccccgaaaag tgccacctga cgtc

FIG. 28

Vargula (463nm) and
Red Italice (613nm)

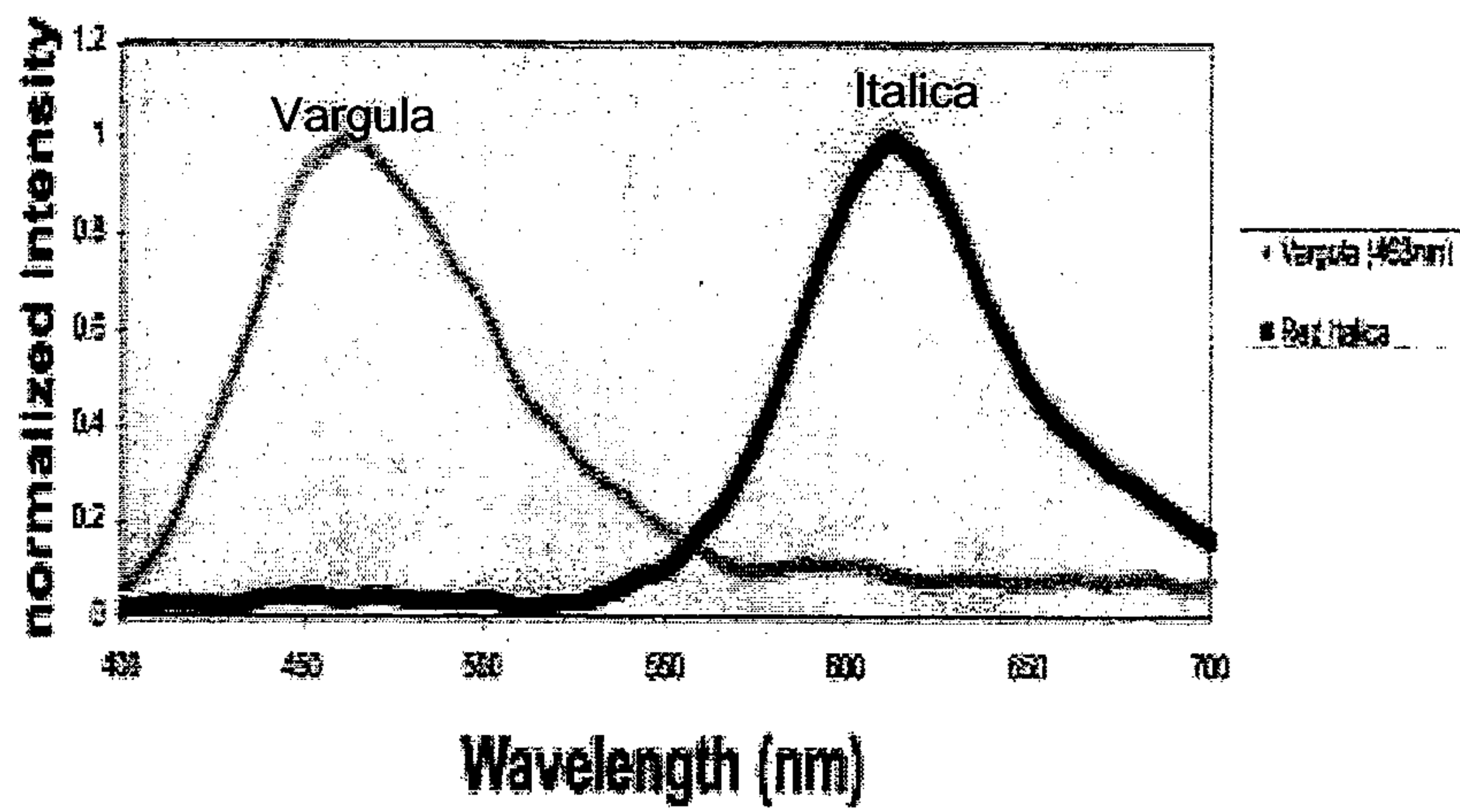


FIG. 29A

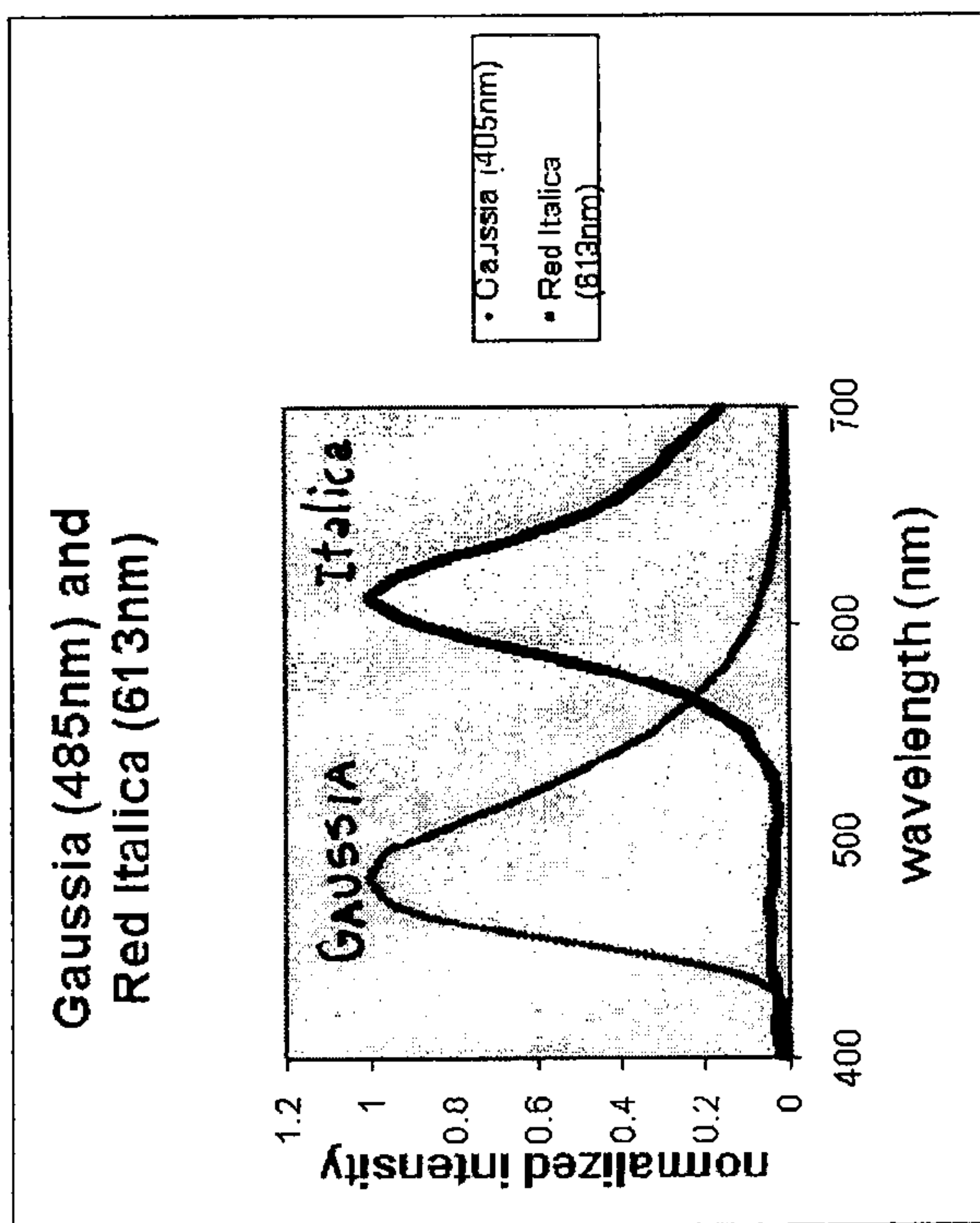


FIG. 29B

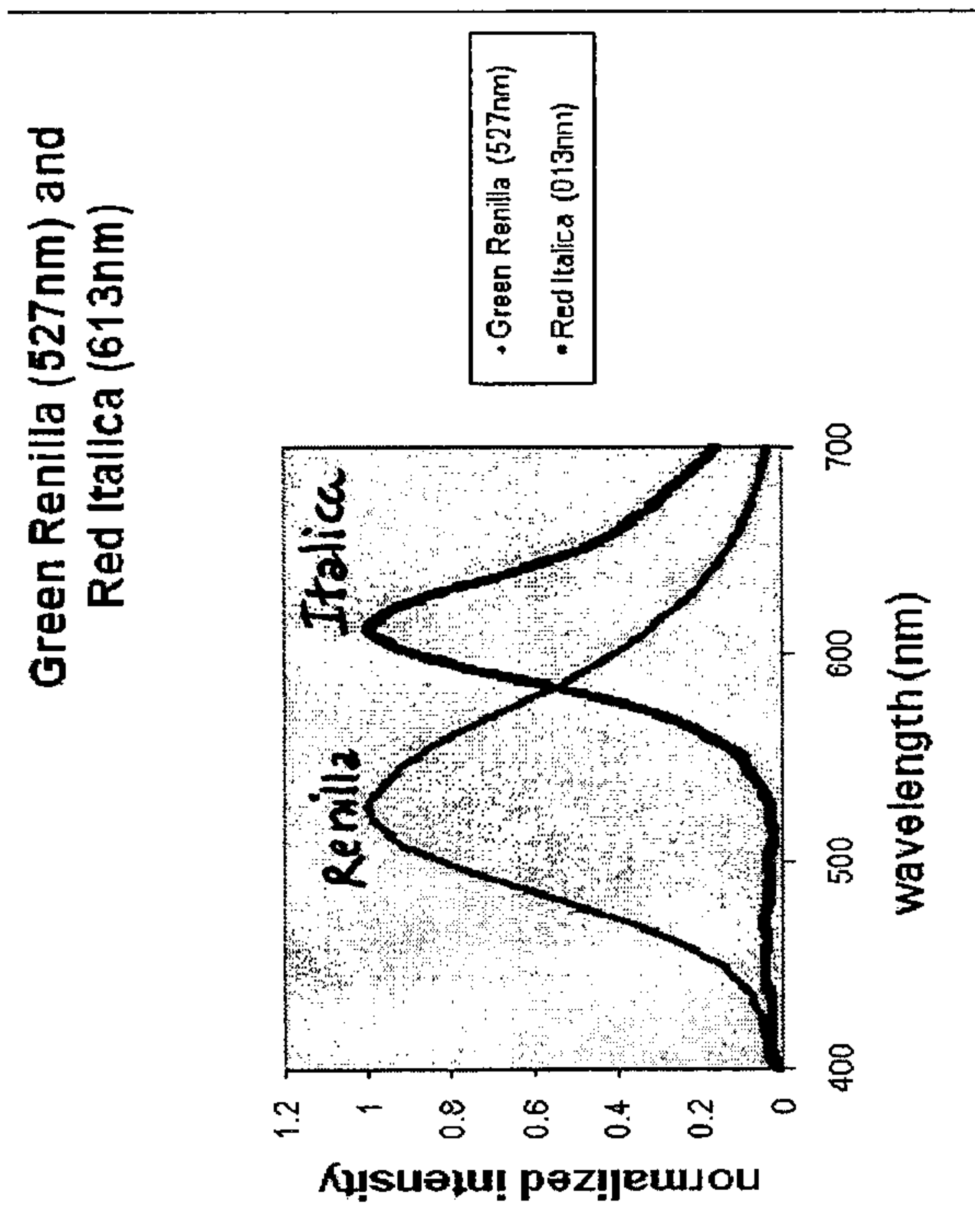


FIG. 30A

A mammalian expression vector expressing Red emitting firefly luciferase (human codon optimized signal) under control of the CMV promoter) (SEQ ID NO: 3)

Size: 6827 bases

```
1   gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61  ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaa ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggtagt gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagtattaa tagtaataa ttacggggtc attagttcal agcccatata
301 tggagttcgg cgttacataa ctacggtaa atggcccggc tggctgaccg cccaacgacc
361 cccgcccatt gacgtcaata atgacgatg tcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccc cttggcagta catcaaggtt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattatgca
601 tcgctattac catggtgatg cggtttggc agtatacaaa tgggcgtgga tagcggttt
661 actcacgggg attccaagt cccacccca ttgacgtcaa tgggagttt ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcga acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841 ctgcttactg gcttatcga aataatacga ctactatag ggagacccaa gcttggctacc
901 gagctcggat ccatggaaac agaaagagaa gaaaacgtt tctacggccc actgccattc
961 taccgatcg aggagggctc tgcggcctc caattgcaca agtacatgca acaatagccc
1021 aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081 tacttcgaca tcacgtgacg actcggcag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagtct tcaatcctgt tctggtggt
1201 cttacatcg gagttacagt cgcgccaact aacgaaatt atacacttag agagctgaac
1261 cacagtctgg ggatagccca acctactatc gtatctcta gcaggaaggc cctgcccaca
1321 gtgcttgagg tgcagaagac cgtgacttc atcaaaacca ttgtatcct ggacagtaag
1381 gtcaactcgc gcggttatga ctgcgtagag accttcaata agaaacacgt cgagctgggc
1441 tttcctgcca cctcattgt gccatcgac gtcaaagacc ggaagacca cattgtctg
1501 cttatgaact cttccggtc cacagggctg ccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctca cgctaaggac cctatatacg gcaatcaggt ggccccaggt
1621 accgctatcc tgactgtct gcctttccac cacggctcg gaatgttca tactttggc
1681 tactttgct cgggttaccg gattgtcatg ctactaagt tcgacgagga gcttttctg
1741 cgcacacttc aggattaca gtgcactaca gtaactctgg tgcggacact gttcgaatt
1801 cttaataggt ctgagctct tgataagtt gacctctca acctgactga aatagccagc
1861 ggtgtgtctc cacttgccaa ggagatcggc gaggctgtg caagaagatt caacctcca
1921 ggcgtccggc agggatagtg actcaccgag actaccagt cctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgtcaaggt gaagattatt
2041 gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctcta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca ctctttata
2221 gtcgaccgcc tgaatctct cattaagat aaaggatacc aagtggcacc agctgaactg
2281 gactgtgtc tctgcaaca cctaactat agagatcgt gttggccgg ggttccgac
2341 agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tctagacta tgtaaactcc caggtggica accacaagcg gctgaggggc
2461 ggcgtgcggt tcttagatga agtcccgaag gggctcacag gaaagatcga cgcgaaagt
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctatctag aaataattct
2581 tactgtcatg ccaagtaaga tgcctttctg tctgcaata gcaggcatgc tggggatgcg
2641 gtggctcta tggcttctga ggcggaaga accagctggg gctctagggg gtatcccac
2701 gcgccctga gggcgcat aagcggcg ggtgtggtgg ttacgcgacg cgtgaccgt
2761 acacttgcca gcgcctagc gcccctctc ttcgcttct tccctctt tctcggcag
2821 ttcgcccgt tccccgta agctctaat cggggcatcc cttaggggt cggatttagt
2881 gcttacggc acctgaccc caaaaaacti gattaggggt atggttcacg tagtgggcca
2941 tgcctgat agacggttt tgcctttg acgttgagt ccacttct taatagtga
3001 ctctgttcc aaactggaac aacctcaac ctaactcgg tctattttt tgattataa
3061 gggattttg ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaattaac
3121 gcgaattaat tctgtggaat gttgtcagt taggtgttgg aaagtccca ggctccccag
3181 gcaggcagaa gtatgcaag catgcatctc aattatgac caaccaggtg tggaaagtcc
3241 ccaggctccc cagcaggcag aagtatgca agcatgcatc tcaattatg agcaaccata
3301 gtcccggccc taactcggc catcccggc ctaactcgc ccagttcgc ctaattctcg
```


FIG. 30B

3361 ccccatggct gactaatttt tttatttat gcagaggccg aggccgcctc tgcctctgag
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttcaa aaagctccc
3481 ggagcttga tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggaggagag gctattcggc
3601 tatgactggg cacaacagac aatcggtcgc tctgatgcc cctgttccg gctgtcagcg
3661 caggggccc cggttcttt tgcaagacc gacctgtccg gtccctgaa tgaactgcag
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc
3781 gacgtgtca ctgaagcggg aagggactgg ctgctattgg gcgaagtgc ggggcaggat
3841 ctctgtcat ctaccttgc tctgcccag aaagtatcca tcatggctga tgcaatgccc
3901 cggctgata cgttcatcc ggctacctgc ccattcgacc accaagcga acatcgcac
3961 gagcgagcac gtactcggat ggaagccggc cttgtcgtc aggatgatc ggacgaagag
4021 catcaggggc tcgcccagc cgaactgtc gccaggctca aggcgcgcac gcccgacggc
4081 gaggatctc tctgaccca tggcgatgcc tcttgcga atatcatgtt ggaatggc
4141 cgtttctg gattcatga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata
4201 gcgttggta cccgtgatat tctgaagag cttggcggcg aatgggctga ccgttctc
4261 gtctttacg gtatcggcg tccgattcg cagcgcacg ctttctatc cttcttgac
4321 gagttctct gagcgggact ctggggctcg aaatgaccga ccaagcgacg cccaacctgc
4381 catcacgaga ttctgattc accgccgct tctatgaaag gttggcttc ggaatcgtt
4441 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttctcggc
4501 accccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcacaatt
4561 tcacaaata agcattttt tcactgcatt ctagtgtgg ttgtccaaa ctcatcaatg
4621 tatctatca tctctgata ccgtcgacct ctactagag cttggcgtaa tcatggtcat
4681 agctgttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa
4741 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc
4801 gctcaactgc cgtttccag tccggaaacc tctctgcca gctgcattaa tgaatcggc
4861 aacgcgggg gagaggggt ttgcgtattg ggcgctctc cgttctctc ctaactgact
4921 cgtcgcgctc ggtcgttcg ctgcggcgag cggatcagc tcaactcaag gcgtaatac
4981 ggttatccac agaatacagg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa
5041 aggccaggaa ccgtaaaaag gccgcgttgc tggcgtttt ccataggctc cggccccctg
5101 acgagcatca caaaaatga cgtcaagtc agagggtggcg aaaccggaca ggactataaa
5161 gataccaggc gtttccccct ggaagctccc tctgctcgc tctgttccg accctgccg
5221 ttaccggata cctgtccgc tttccctt cgggaagcgt ggcgcttct caatgctcac
5281 gctgtagga tctcagttcg gtgtaggtcg tctctcaa gctgggctgt gtgcacgaac
5341 cccccgtca gcccgaccgc tgcgcttat ccgtaacta tctcttgag tccaaccgg
5401 taagacacga ctatcgcca ctggcagcag ccaactgtaa caggattagc agagcgagg
5461 atgtaggcgg tctacagag tcttgaagt ggtggcctaa ctacggctac actagaagga
5521 cagtattgg tatctgcgt ctgctgaagc cagttacct cggaaaaaga gttgtagct
5581 cttgatccg caacaaacc accgctgga gcggtggtt tttgttgc aagcagcaga
5641 ttacgcgcag aaaaaagga tctcaagaag atccttcat ctttctac gggctgacg
5701 ctcaatgaa cgaaaactca cgttaaggga tttggtcat gagattatca aaaaggatc
5761 tcaactagat cctttaaat taaaaatgaa gtttaaatc aatctaaagt atatagagt
5821 aaacttggc tgacagttac caatgctaa tcaatgaggc acctatctca gcgatctgc
5881 tattcgttc atccatagt cctgactcc ccgtcgtga gataactacq atacgggagg
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggtccag
6001 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtgtt cctgcaact
6061 tatccgctc catccagtct attaatgtt gccgggaagc tagagtaagt agttccag
6121 ttaatagtt gcgcaactt gttgccattg ctacaggcat cgtgggtgca cgtcgtcgt
6181 ttggtatggc tcaatcagc tccggttccc aacgatcaag gcgagttaca tgatcccca
6241 tgtgtgcaa aaaagcgggt agctccttcg gctcctccat cgttgcaga agtaagtgg
6301 ccgcatggt atcaactatg gttatggcag cactgcataa tctcttact gcatgcat
6361 ccgtaagatg ctttctgtg actggtgagt actcaacaa gtcattctga gaatagtga
6421 tgcggcgacc gagttgctt tccccggc caatacggga taataccgc ccatagca
6481 gaacttaaa agtgctcatc attgaaaac gttctcggg gcgaaaactc tcaaggatc
6541 taccgctgt gagatccagt tctatgtaac ccactcgtc acccaactga tctcagcat
6601 ctttacttt caccagcgt tctgggtgag caaaaacagg aaggcaaat gccgcaaaa
6661 aggaataag ggcgacacgg aatgttga tactcact cttctttt caatattat
6721 gaagcattt tcagggtat tctctatga gcgatacat attgaaatg atttagaaa
6781 ataaacaat aggggttccg cgcacattc cccgaaaagt gccacctgac gtc

FIG. 31A

Sequence and Features of pCMV GrFLUC Vector:

A mammalian expression vector expressing human codon optimized green firefly luciferase (*Luciola Italica*) under control of the CMV promoter (SEQ ID NO: 4)

Size: 6827 bases

pCMV-GrFLuc (6827 bp)

CMV promoter bases: 209-863

Green emitting firefly luciferase gene: 907-2560

T7 promoter bases: 1827-1845

Polylinker bases: 1852-1870

SP6 promoter: 2576-2593

Synthetic polyadenylation site: 2560-2604

SV40 promoter bases: 3145-3480

SV40 origin of replication: bases: 3259-3344

Neomycin ORF : bases 3516- 4310

SV40 PolyA: bases 4365-4737

ColE1 origin: bases 3934-4607

Ampicillin ORF: bases 4752-5612

```

1  gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaate tgctctgatg
61  ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggctgct gagtagtgcg
121  cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181  ttagggtag  gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241  gattattgac tagtattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301  tggagtccg  cgttacataa ctacggtaa atggcccgc tggctgaccg cccaacgacc
361  cccgcccatt gacgtcaata atgacgtatg ttccatagt aacgccaata gggactttcc
421  attgacgtca atgggtggac tattacggt aaactgcca cttggcagta catcaaggtg
481  atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541  atgcccagta catgacctta tgggacttcc ctacttgcca gtacatctac gtattagtca
601  tcgctattac catggtgatg cggttttggc agtacatcaa tgggctgga tagcggttg
661  actcacgggg attccaagt ctccaccca ttgacgtcaa tgggagttg tttggcacc
721  aaaatcaacg ggactttcca aaatgctgta acaactccgc cccattgacg caaatgggcg
781  gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841  ctgcttactg gcttatcga aaataacga ctactatag ggagaccaa gcttggtagc
901  gagctcggat ccatggaaac agaaagagaa gaaaacgtg tctacggccc actgccatc
961  tacccgatcg aggagggctc tgcggcacc caatgcaca agtacatgca acaatagccc
1021  aagctcggcg ccatcgctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081  tacttcgaca tcacgtgcag actcgccgag gctatgaaga actacggcat gaagccagaa
1141  ggacacatcg ctctcttag cgagaactgc gaagagtct tcttctgt tctggctggt
1201  ctttacatcg gagttacagt cgcgccaact aacgaaatt atacacttag agagctgaac
1261  cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaggc cctgcccaca
1321  gtgcttgagg tgcagaagac cgtgactgac atcaaaacca ttgtatcct ggacagtaag
1381  gtcaacttcg gcggttatga ctgcgtagag acctcatta agaaacacgt cgagctgggc
1441  tttctgcca cctcattgt gccatcgac gtcaagacc ggaagacca cattgtctg
1501  cttatgaact ctccggctc cacagggtc cccaaaggag tagagatcac tcacaggccc
1561  ctggtcacga gattctctca cgtaaggac cctatatacg gcaatcaggt ggcccaggt
1621  accgctatcc tgactgtcat cctttccac cagccttcg gaatgagcac tactttgggc
1681  tactttgctt gcggttaccg gattgtcatg cttactaagt tcgacgagga gcttttctg
1741  cgcacacttc aggattacaa gtgcactagc gtaatcctgg tgcgacact gttcgcaatt
1801  ctaaataggt ctgagctcct tgataagttt gaccttcta acctgactga aatagccagc
1861  ggtggtgctc cacttgcca ggagatcggc gaggctgtt caagaagatt caacctcca
1921  ggcgtccggc agggatatgg actcaccgag actaccagt cctttatcat cactcctaag
1981  ggcgacgaca agccgggagc cagcggcaag gtcgtgctc tgtcaaggt gaagattatt
2041  gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101  ccatctctta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161  gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca cttcttata
2221  gtcgaccgcc tgaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg
2281  gactctgtgc tctgcaaca ccctaacatt agagatgctg gtgtggccgg ggttcccagc
2341  agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401  gagaaagaaa tcgtagacta tgtaactcc cagggtgtca accacaagcg gctgaggggc
2461  ggctgctggt tcgtagatga agtcccaag gggctcacag gaaagatcga cgcgaaagt

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FIG. 31B

2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctagaataa ttctactgt
2581 catgccaagt aagatgcttt tctgtgctgc aatagcaggc atgctgggga tgcggtgggc
2641 tctatggctt ctgaggcggga aagaaccagc tggggctcta gggggtatcc ccacgcgcc
2701 tgtagcggcg cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt
2761 gccagcgccc tagcggccgc tccittcgtt ttctccctt ccttctcgc cacgttcgcc
2821 ggctttcccc gtcaagctct aatcggggc atcccttag ggttccgatt tagtgctta
2881 cggcacctcg acccaaaaa actigattag ggtgatggtt cacgtagtgg gccatcgccc
2941 tgatagacgg ttttcgccc ttgacgttg gagtccactt tcttaatag tggactctg
3001 ttccaaactg gaacaact caacctatc tccgtctatt ctttgattt ataagggtt
3061 ttggggattt cggcctattg gtaaaaaat gagctgattt acaaaaaatt taacgcgaat
3121 taattctgtg gaatgtgtgt cagttaggtt gtggaaagtc cccaggctcc ccaggcaggc
3181 agaagtatgc aaagcatgca tctcaattag tcagcaacca ggtgtggaaa gtccccaggc
3241 tccccagcag gcagaagat gcaaagcatg catctcaatt agtcagcaac catagtcccg
3301 ccctaactc cgccatccc gccctaact cggccagtt cggccattc tccgcccatt
3361 ggctgactaa tttttttat ttatgcagag gccgaggccg cctctgcctc tgactatc
3421 cagaagtagt gaggaggctt tttggaggc ctaggctttt gcaaaaagct cccgggagct
3481 tctatatcca tttcggatc tgatcaagag acaggatgag gatcgtttcg catgattgaa
3541 caagatgat tgcacgcagg ttcccgccg gcttgggtgg agaggctatt cggctatgac
3601 tgggcacaac agacaatcgg ctgctctgat gccgccgtgt tccggctgtc agcgcagggg
3661 cgcccggtc ttttgtcaa gaccgacctg tccggtgccc tgaatgaact gcaggacgag
3721 gcagcgggc tctgtggct ggccacgacg ggcgttcctt gcgagctgt gctcagctt
3781 gtcactgaag cgggaaggga ctggctgcta tgggcgaag tccggggca ggtctcctg
3841 tcatctacc ttgctcctgc cgagaaagta tccatcatgg ctgatgcaat gcggcggctg
3901 catacgctt atccggctac ctgcccattc gaccaccaag cgaacatcg catcagcga
3961 gcacgtactc ggatggaagc cggctctgtc gatcaggatg atctggacga agagcatcag
4021 gggctcgcgc cagccgaact gttcggcagg ctcaaggcgc gcattgcccga cggcgaggat
4081 ctctctgta cccatggcga tgcctgctg ccgaatatca tggtggaata tggccgctt
4141 tctggattca tgcactgtgg ccggctgggt gtggcggacc gctatcagga catagcgtt
4201 gctaccctg atattgctga agagcttggc ggcgaatggg ctgaccgctt cctcgtgctt
4261 tacggatcg ccgctcccga ttcgacgagc atgccttct atgccttct tgacgagtc
4321 ttctgagcgg gactctgggg ttgaaatga ccgaccaagc gacgccaac ctgcatcac
4381 gagattcga ttccaccgcc gccttctatg aaagggtggg ctccggaatc gtttccggg
4441 acgcccggctg gatgatctc cagcgcgggg atctcatgct ggagtctc gccacccca
4501 acttcttat tgcagctat aatggttaca aataaagcaa tagcatcaca aattcaca
4561 ataaagcatt ttttactg cattctagt gtggtttgc caaactcacc aatgtaact
4621 atcatgtctg taccctgc acctctagct agagcttggc gtaatcatgg tcatagctgt
4681 ttctgtgtg aaattgttat ccgctcaca ttccacaca catacagacc ggaagcataa
4741 agttaaagc ctgggtgccc taatgagtga gtaactcac ataatgctg ttgcgctcac
4801 tccccgctt ccagtcggga aacctgctg gccagctgca ttaatgaatc ggccaacgcg
4861 cggggagagg cggtttgcgt attgggcgct ctccgctc ctgctcact gactcgtgc
4921 gctcggctgt tggctgccc cgagcggat cagctcactc aaaggcggia atacggtat
4981 ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca
5041 ggaaccgtaa aaaggccgcg ttgctggcgt tttccatag gctccgccc cctgacgagc
5101 atcaaaaaa tgcagctca agtcagaggt ggcgaaacc gacaggacta taaagatacc
5161 aggcgttcc cctggaagc tccctcgtc gctctctgt tccgacctg ccgcttaccg
5221 gatacctgc cgcttctc cctcgggaa cgttggcgtt ttctaatgc taccgctga
5281 ggtatctcag tccggtgtag gtcgttcgct ccaagctggg ctgtgtgac gaacccccg
5341 ttacggcga ccgctgccc ttatccgta actatctct ttagtccaac ccgtaagac
5401 acgactatc gccactgca gcagccactg gtaacaggat tagcagagcg aggtatgtag
5461 gcggtgctac agagtcttg aagtgtggc ctaactacgg ctactacta aggacagat
5521 ttggtatctg cgtctgctg aagccagta ccttcgaaa aagagtgtgt agctcttgat
5581 ccggcaaca aaccaccgt gtagcgggt gttttttt ttgcaagcag cagattacgc
5641 gcagaaaaa aggatctca gaagatcct tcatctttc tacggggtct gacgctcagt
5701 ggaacgaaa ctacgtaaa gggatttgg tcatgagatt atcaaaaagg atctcacct
5761 agatcctttt aattaaaaa tgaagtitta aatcaacta aagtataat gagtaactt
5821 ggtctgacag ttaccaatgc ttaactagt aggcacctc ctacagctc tctctattc
5881 gttcatccat agttgctga ctcccctgc ttagataac tacgatacgg gagggcttac
5941 catctggccc cagtctgca atgataccgc gagaccacg ctaccggct ccagattat
6001 cagcaataaa ccagccagcc ggaaggccg agcgcagaag tggctctgca actttatccg
6061 cctccatca gtctattaat tgtgcccgg aagctagagt aagtagtctg ccagtaata
6121 gttgcaaa cgttggcc attgctacag gcactgtgt gtcacgctc tcttttgta

FIG. 31C

6181 tggcttcatt cagctccggt tcccaacgat caaggcgagt tacatgatcc cccatgttgt
6241 gcaaaaaagc ggtagctcc ttcggctctc cgatcggtgt cagaagtaag ttggccgcag
6301 tggatcact catggtatg gcagcactgc ataattctct tactgtcatg ccatccgtaa
6361 gatgctttc tgtgactggt gactactcaa ccaagtcatt ctgagaatag tgtatgcggc
6421 gaccgagttg ctctgcccg gcgcaatac gggataatac cgcgccacat agcagaactt
6481 taaaagtgt catcattgga aaacgttctt cggggcgaaa actctcaagg atctaccgc
6541 tgttgagatc cagttcgatg taaccactc gtgcacccaa ctgatcttca gcactttta
6601 cttcaccag cgttctggg tgagcaaaaa caggaaggca aaatgccgca aaaaaggaa
6661 taaggcgac acggaaatgt tgaatactca tactcttct tttcaatat tattgaagca
6721 ttatcaggg ttattgtctc atgagcggat acatattga atgtattag aaaaataaac
6781 aaataggggt tccgcgcaca ttccccgaa aagtccacc tgacgtc

FIG. 32A

The sequence of the CMV expression vector expressing human codon optimized Vargula luciferase under control of the CMV promoter (not the vargula luciferase sequence) is in bold.

CMV promoter bases: 209-863
 Vargula luciferase gene: 907-
 T7 promoter bases: 864-882
 Polylinker bases: 889-907

gacggatcgggagatctcccgatccctatggctgactctcagtaacaatc tgctctgatgccgcatagtaagccagatctgctccctgcttgtgtgtt
 ggaggtcgtgtagtgctgagcaaaattaaagctacaacaaggcaag gcttgaccgacaattgcatgaagaatctgcttagggtaggcgtttgctg
 ctgcttcgcatgtacggccagatatacgcgttgacattgattattgac tagttattaatagtaatacaattacggggcattagttcatagcccatata
 tggagttccgcgttacataacttacggtaaatggccgcctggctgaccg cccaacgacccccgccattgacgtcaataatgacgtatgttcccatagt
 aacgccaatagggactttccattgacgtcaatgggtggactattacgggt aaactgccactggcagtcacatcaagtgtatcatatgccaagtacgccc
 cctattgacgtcaatgacggtaaatggccgcctggcattatgccagta catgacctatgggactttcctacttggcagtcacatctacgtattagta
 tcgctattaccatgggtgatgcggtttggcagtcacatcaatgggcgtgga tagcggttgactcacggggatticcaagtcctccacccattgacgtcaa
 tgggagttgtttggcaccaaaatcaacgggactttcaaaatgtcgtgta acaactccgccccattgacgcaaatgggcgttaggcgtgtacgggtgggag
 gtctatataagcagagctctctgctaactagagaacccactgcttactg gcttatcgaaataatagactcactatagggagaccaagcttgggtacc
 gagctc **ATGAAGATAATTATCCTTTCTGTGATTCTGGCTTACTGTGTTAC**
AGTGAATTGTCAGGATGCATGTCCAGTAGAGGCGGAACCGCCATCTTCTA
CCCCGACCGTACCAACCTCCTGCGAAGCTAAAGAAGGGGAGTGCATCGAT
ACAAGGTGCGCTACCTGCAAACGGGATATCCTGTCCGACGGACTTTGCGA
AAATAAACCCGGAAGACCTGCTGTGCAATGTGTGTCAGTATGTCATCGAAT
GCCGGTTCGAGGCCCGGTTATTTAGAACATTTTACGGTAAACGGTTT
AATTTCCAGGAACCCGGCAAATACGTAAGTGGCTCGCGGCACCAAGGGTGG
CGACTGGAGCGTCACCCTGACAATGGAAAACCTGGACGGGCAGAAAGGAG
CCGTGCTTACTAAACTACCTGGAGGTGGCGGGAGACGTAATTGACATC
ACTCAGGCAACGGCTGACCCAATAACCGTGAACGGAGGAGCTGATCCCGT
GATTGCAAACCTTTCACTATTGGCGAGGTCACGATTGCCGTCGTCGAAA
TTCCAGGCTTCAACATCACAGTATCGAGTTCCTTCAAGCTGATCGTCATT
GATATCCTCGGCGGACGGTCCGTTCCGCATCGCACCTGACACAGCCAACAA
GGGCTGATCTCTGGCATTGTTGGTAACCTTGAAATGAATGATGCTGATG
ACTTCACAACGGACGCCGACCAACTGGCCATTCAACCTAATATCAACAAA
GAGTTTGATGGATGTCCCTTTACGGAAATCCTTCAGACATCGAATACTG
CAAAGGCCTCATGGAACCGTACCGGGCCGTTTGCAGAAATAACATCAACT
TCTACTATTATACTCTGAGCTGCGCATTGTCATACTGTATGGGCGGTGAG
GAGAGAGCCAAACATGTGCTTTTCGACTATGTGGAGACCTGCGCCGCCCC
GGAGACTCGCGGTACCTGCGTCCTGAGCGGCCATACCTTCTATGACACCT
TCGATAAGGCTAGGTACCAGTTCGAAGGGCCTTGCAAAGAGCTCCTGATG
GCCGCAGATTGTTACTGGAACACTTGGGACGTCAAAGTTTCCCATCGGGA
CGTAGAGAGCTACACGGAAGTTGAGAAGGTGACCATCAGGAAGCAGAGTA
CCGTGCTAGACCTGATCGTCGACGGCAAGCAGGTAAGGTAGGAGGCGTG
GACGTTAGTATTCCGTATTCTTCTGAAAATACGAGCATCTACTGGCAGGA
TGGAGACATTCTGACAACCGCCATCCTTCCAGAAGCTCTGGTGGTGAAGT
TTAACTTCAAGCAGCTGCTGGTAGTGACATTTCGCGACCCATTTCGACGGG
AAAACCTGTGGGATTTGCGGCAACTACAACCAGGACTCAACTGACGATTT
CTTTGACGCCGAAGGGGCTTGCCTCTTACCCCAAATCCGCCTGGATGCA
CCGAAGAGCAAAGCCTGAAGCGGAACGGCTGTGCAATTCAGTGTGAT
TCTTCAATAGATGAGAAATGCAACGTGTGTTACAAACCTGACCGCATCGC
ACGCTGCATGTATGAGTATTGCCTGAGAGGTCAACAAGGGTTCTGCGATC
ACGCGTGGGAATTTAAGAAAGAATGCTACATAAAGCACGGGGATACATTG
GAGGTGCCGCCAGAATGCCAGTAGTctagaaataaacttactgtcatgccaagtaagatgcttttctgtgctgcaat
 agcaggcatgctgggatcgggtgggctctatggcttctgaggcgaaag aaccagctggggcttaggggtatccccacgcccctgtagcggcgc
 taagcggcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgc agcgccttagcggcctctcttctgctttcttcccttcttctgcccac
 gttcgcggcgtttccccgtcaagcttaaatcgggcatccctttagggt tccgatttagtctttacggcacctcgacccccaaaaaacttgattagggt
 gatgggtcacgtatggccatcgcctgatagacggtttttgcccctt gacgttgagtcacgcttcttaatagtgactctgttccaaactggaa
 caaacctcaaccctatctcggctattctttgattataagggaatttg gggatttcggcctattggttaaaaaatgagctgatttaacaaaaattaa
 cgcgaatfaattctgtggaatgtgtgctagtaggtgtgaaagtcccc aggctccccaggcaggcagaagatgcaaagcatgcatctcaattagtca
 gcaaccaggtgtgaaagtccccaggctccccagcaggcagaagatgca aagcatgcatctcaattagtcagcaacctagtcgccccctaaactccgc
 ccatccccctaaactccgcccagttccgcccatttccgcccattggc tgactaattttttatfatgagaggccgaggccgctctgctctga
 gctattccagaagtagtgaggaggctttttggaggcctagcctttgca aaaagctccccggagctgtatatacattttcgatctgatcaagagaca
 ggataggatcgtttcgcattgattgaacaagatggattgcacgcagggtc tccggccctgggtggagaggctattcggctatgactgggcacaacaga

FIG. 32B

caatcggctgctctgatgccccgtgtccggctgtcagcgcaggggccc cgggtcttttgtcaagaccgacctgtccgggtgccctgaatgaactgca
ggacgagggcagcgggtatcgtggctggccacgacgggcttccctgcg cagctgtgctcagcgtgtcactgaagcgggaaggactggctgctattg
ggcgaagtccggggcaggatctcctgtatctcacctgtcctgcccga gaaagtatccatcatggctgatgcaatcggcggctgcatacgtgatc
cggctacctgccattcgaccaccaagcgaacatcgcacgagcgagca cgtactgggatggaagccggtcttctgatcaggatgatctggacgaaga
gcatcaggggctcgcgccagccgaactgtccagggctcaaggcgcgca tgcccagcggcaggatctcgtcgtgacctggcgatgctgcttggcc
aatatcatggtggaaaatggccgcttttctgattcatcactgtggccg gctgggtgtggcggaccgtatcaggacatagcgttggctaccctgata
ttgctgaagagcttggcggcgaatgggctgaccgcttctcgtgctttac ggtatcggcctcccattcgcagcgcacgccttctatgccttctga
cgagttctctgagcgggactctggggctcgaatgaccgaccaagcgac gcccaacctgccatcacgagattcattccaccgcccttctatgaaa
ggttgggcttcggaatcgtttccgggacgccggctggatgatctccag cgcggggatctatgctggagttctcggccaccccaactgtttattgc
agcttataatggttacaataaagcaatagcatcacaatttcacaaata aagcatttttctactgcattctagtgtgttggtttgcacaaactcaat
gtatctatcatgtctgtatacctgcacctctagctagagcttggcgta atcatggtcatagctgttctgtgtgaaattgtatccgctcacaattc
cacacaacatacagaccggaagcataaagtgtaaagcctggggtgcctaa tgagttagtaactcacattaattgcttgcgctcactgccgcttcca
gtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgg ggagaggcgggttgcgtattggcgctcttccgcttctcgtcactgac
tcgctcgcctcggctcgttggctcggcgagcggatcagctcactcaaa ggcggtaatacgggttatccacagaatcaggggataacgcaggaaagaaca
tgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcttg ctggcgttttccataggctccgccccctgacgagcatcaaaaaatcg
acgctcaagtcagaggtggcgaacccgacaggactataaagataccagg cgtttccccctggaagctccctcgtcgcctcctcttccgacctgccg
cttaccggatacctgtccgcttctccttccgggaagcgtggcgcttcc tcaatgctcacgctgtaggtatctcagttcgggtgtagctcgtcctca
agctgggctgtgtgcacgaacccccctcagcccagccgtcgcctta tccgtaactatcgtctgagtcacaacccgtaagacacgacttatgcc
actggcagcagccactgtaacaggattagcagagcggatgtaggcg gtgtacagagttcttgaagtgggtggcctaactacggctacactagaagg
acagtattggtatctgcgctctgctgaagccagttacctcggaaaaag agttggtagctctgatccggcaacaaaccaccgctggtagcgggtgt
ttttgttgaagcagcagattacgcgcagaaaaaaggatctcaagaa gatccttctatcttctacggggtctgacgctcagtggaacgaaaactc
acgttaagggttttggatgagattcaaaaaaggatcttccactaga tcttttaaaatlaaaatgaagttttaaataatctaaagtatatatgag
taaacttggctgacagttaccaatgcttaatcagtgaggcacctatctc agcgatctgtctattcgttccatcatagttgcctgactcccgtcgtgt
agataactacgatacgggagggttaccatctggcccagtgctgcaatg ataccgcgagaccacgctcaccggctccagatttatcagcaataacca
gccagccggaaggccgagcgcagaagtgtcctgcaactttatccgct ccatccagctatataattgttggcgggaagctagagtaagtagttcgcca
gtaatagtttgcgcaacgttgttccattgctacaggcatcgtgggtgc acgctcgtcgttggatggctcattcagctccgggtcccaacgatcaa
ggcgagttacatgatccccatgtgtgcaaaaaagcgggttagctcctc ggtcctccgacgtgtgcagaagtaagtggccgcagttatcactcat
ggttatggcagcactgcataattcttactgtcatgccatccgtaagat gctttctgtgactggtgagtactcaaccaagtcattctgagaatagtg
atgcggcgaccgagttgcttggccggcgtcaatacgggataataccgc gccacatagcagaacttlaaaagtctatcattgaaaacgttctcgg
ggcgaactctcaaggatcttaccgctgttgagatccagttcgatgtaa cccactcgtgcacccaactgatcttcagcatctttactttaccagcgt
ttctgggtgagcaaaaacaggaaggcaaatgccgcaaaaaagggaataa gggcgacacggaaatgtgaatactcactcttcttttcaatattat
tgaagcatttatcagggttattgtctcatgagcggatacatattgaatg taittagaaaaataacaaataggggttccgpcacatttcccgaag
tgccacctgacgtc

MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/238,146, filed on Aug. 29, 2009, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention concerns the field of luciferase reporters useful in biological and biochemical assays.

BACKGROUND OF THE INVENTION

Luciferases are enzymes that catalyze reactions that emit light. Luciferases are named according to their source organisms such as beetles (firefly) or marine organisms. Examples of bioluminescent marine animals include: *Renilla*, also known as sea pansies, which belong to a class of coelenterates known as the anthozoans. In addition to *Renilla*, other representative bioluminescent genera of the class Anthozoa include *Cavarnularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, and *Parazoanthus*. All of these organisms are bioluminescent and emit light as a result of the action of an enzyme (luciferase) on a substrate (luciferin) under appropriate biological conditions.

Different luciferases have different properties with regard to substrate specificity and intensity of light emission and stability of the bioluminescent signal, which is commonly measured by a luminometer. Luciferases are useful as transcriptional reporter genes and in imaging reporter gene expression in living subjects and many other applications in molecular biology.

Certain luciferases, such as those that utilize *cypridina* luciferin (vargulin) as a substrate, can be useful reporters because of their strong luminescent signal and the fact that they are secreted in the native form. However *cypridina* luciferin (vargulin) is very difficult to synthesize (usually involving an 18-step chemical synthesis). The limiting supply and the cost of the material have made the assay difficult to commercialize.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides modified luciferases, methods of making modified luciferases, and methods of using modified luciferases.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Luciola Italica* (also referred to as *L. Italica*) luciferase. In a further aspect, the modified *L. Italica* luciferase shows increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In an embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that shows an approximately 1000-fold increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a red-emitting luciferase with an emission maximum of approximately 617 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is human codon-optimized.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a green-emitting luciferase with an emission maximum of approximately 550 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that includes a secretory signal at its amino terminal end. In a still further embodiment, the secretory signal is a chymotrypsinogen secretory signal.

In one aspect, the present invention provides assays utilizing any of the modified luciferases discussed herein. In a further aspect, the assays are multiplexed reporter assays.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Renilla* luciferase. In a further aspect, the modified *Renilla* luciferase shows increased activity and stability over a native human codon optimized *Renilla* luciferase.

In an exemplary embodiment the present invention provides a modified *Renilla* luciferase that is a green-emitting *Renilla* luciferase.

In a further embodiment and in accordance with any of the above, the invention provides a modified *Renilla* luciferase that includes a secretory signal at its amino terminal end.

In one aspect, the present invention provides multiplexed luciferase assays comprising at least two different luciferase reports, where the at least two different luciferase reporters emit at two different wavelengths and/or utilize different substrates.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows data for relative luciferase stability for a *Cypridina* assay conducted using reagents without sodium chloride (VLAR-1) and with sodium chloride (VLAR-1 with sodium chloride).

FIG. 2 shows data for time course of activity in a *Cypridina* assay using 5 μ l of sample (FIG. 2A) or 20 μ l of sample (FIG. 2A).

FIG. 3A-B shows the sequence of a green *Renilla* luciferase plasmid (SEQ ID NO: 1).

FIG. 4 shows the sequence of a modified red firefly luciferase with a secretory signal (SEQ ID NO: 2).

FIG. 5 shows data from a *Cypridina* luciferase assay in varying concentrations of sodium chloride.

FIG. 6 shows data from a *Renilla* luciferase assay with and without stabilizer (NP40).

FIG. 7 shows data comparing luciferase activity of native human codon optimized *Renilla* luciferase and a mutant *Renilla* luciferase of the invention.

FIG. 8 shows data comparing luciferase activity of human codon optimized and non-human codon optimized red-emitting *L. Italica* luciferase.

FIG. 9 shows data comparing luciferase activity of human codon optimized and non-human codon optimized green-emitting *L. Italica* luciferase.

FIG. 10 shows data comparing luciferase activity of intracellular red-emitting *L. Italica* luciferase and secreted red-emitting *L. Italica* luciferase.

FIG. 11 shows data comparing luciferase activity of secreted red *L. Italica* luciferase in the lysate and the supernatant from HEK293 cells.

FIG. 12 shows kinetics of luciferase activity in (A) Red *Luciola* luciferase, (B) *Guassia* luciferase, (C) *Cypridina* luciferase, and (D) Green *Renilla* luciferase.

FIG. 13 shows emission spectra from (A) a double reporter assay with *Vargula* and Red *Italica* luciferases and (B) a triple reporter assay with *Vargula*, Green *Renilla* and Red *Italica* Luciferases.

FIG. 14 shows kinetics data of a *Gaussia* luciferase assay using a GAR-1 reagent.

FIG. 15 shows data comparing stabilities of *Gaussia* luciferase assays using the GAR-2 reagent are in the presence of a stabilizer (FIG. 15A) and in the absence of a stabilizer (FIG. 15B).

FIG. 16 shows data related to relative luciferase activity of a firefly luciferase assay.

FIG. 17 shows data of relative luciferase activity of a *Cypridina* luciferase assay.

FIG. 18 shows data comparing luciferase activity of a modified *Vargula* luciferase of the invention in the lysate and the supernatant from mammalian cells.

FIG. 19 shows kinetic data for luciferase activity in a *Cypridina* luciferase assay.

FIG. 20 shows data comparing relative luciferase activity of Green *Renilla* luciferase in the absence (top panel) and presence (bottom) of a stabilizer.

FIG. 21 shows data from a firefly luciferase assay in the presence (square) and absence (diamonds) of a stabilizer.

FIG. 22 shows data from a dual assay of the invention utilizing firefly and *Cypridina* luciferases.

FIG. 23 shows data from a dual assay of the invention utilizing *Cypridina* (Panel A) and *Renilla* (Panel B) luciferases.

FIG. 24 shows emission spectra from a dual assay of the invention utilizing *Vargula* and Green *Renilla* luciferases.

FIG. 25 shows data from a triple assay of the invention utilizing *Cypridina*, firefly and *Gaussia* luciferases.

FIG. 26 shows emission spectra from a triple assay of the invention utilizing *Cypridina*, Green *Renilla* and Red *Italica* luciferases.

FIG. 27 shows the sequence of a red firefly luciferase of the invention (SEQ ID NO: 5).

FIG. 28 shows emission spectra from a dual assay of the invention utilizing *Vargula* and Red *Italica* luciferases.

FIG. 29 shows emission spectra from a dual assay of the invention utilizing (A) *Gaussia* and Red *Italica* luciferases and (B) Green *Renilla* and Red *Italica* luciferases.

FIG. 30 shows the sequence of a red emitting firefly human codon optimized luciferase of the invention (SEQ ID NO: 3).

FIG. 31 shows the sequence of a human codon optimized green firefly luciferase of the invention (SEQ ID NO: 4).

FIG. 32 shows the sequence of a human codon optimized *Vargula* luciferase of the invention (SEQ ID NO: 6).

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing devices, formulations and methodologies which are described in the publication and which might be used in connection with the presently described invention.

Note that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a polymerase” refers to one agent or mixtures of such agents, and reference to “the method”

includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention. It will be apparent to one of skill in the art that these additional features are also encompassed by the present invention.

Overview

The present invention provides modified luciferases and/or combinations of luciferases, and methods of utilizing those luciferases in reporter gene assays. In addition, the invention provides reagents that provide increased stability and activity in assays using luciferase reporters.

The present invention provides modified (also referred to herein as “mutant” or “variant”) luciferases showing improved activity over wildtype luciferases or other modified luciferases known in the art reported to have improved properties for reporter gene assays or in vivo imaging applications. As used herein, “wildtype luciferases” refers to any luciferase that occurs in nature.

In certain aspects, the present invention provides modified luciferases that show brighter luminescence when expressed in mammalian cells as compared to the luminescence seen when wildtype luciferases are expressed in mammalian cells. The present invention also provides a method of expressing luciferase as a very bright intracellular reporter (not secreted) by sequence modification to increase its utility as an intracellular reporter in multiplexed assays and for imaging applications. The present invention also provides a composition for assays utilizing luciferases that lowers the cost and increases the efficiency and sensitivity of the assay by altering the reaction conditions such that high luminescence is produced using significantly less amount of luciferin.

The present invention further provides reagents for assays utilizing modified luciferases of the invention as well as mammalian expression vectors expressing secreted and intracellular luciferases.

In further embodiments the present invention provides sequence modifications (human codon optimization) to nucleotides encoding luciferases which result in an approximately 1000-fold increase in luciferase expression in transfected mammalian cells compared to the non-human codon optimized versions of these genes.

In further embodiments, the invention provides novel secreted reporter modified luciferases that are about 5 to about 35 fold brighter than wildtype luciferases. Such luciferases are used in accordance with the present invention as stand alone reporters or in multiplexed luciferase assays in combination with one or more other luciferases. As will be appreciated, combinations of luciferases for multiplexed assays of the invention can include both wildtype and modified luciferases.

In further aspects, the present invention provides assay compositions for measurement of modified luciferases of the invention as single luciferase assay formats. In further aspects of the invention, assay compositions are provided that enable simultaneous measurement of at least two different reporters in cell lysates or supernatants using a single assay solution. The luciferase activities of multiple reporters are analyzed by exploiting spectral differences in the emission maxima of the different luciferases.

Improved luciferases used in the present invention include without limitation: (i) a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (ii) a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (iii) a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used); (iv) *Vargula* luciferase that has been modified at the C-terminal end with a KDEL sequence (endoplasmic reticulum retention signal) so that it is expressed intracellularly-VLuc-KDEL; (v) a modified secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc) which is brighter and more stable than native *renilla reniformis* luciferase; (vi) a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *renilla* luciferase; (vii) a *Gaussia* luciferase (emission max 482 nm) either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL).

Luciferases of the Invention

Modified luciferases of the present invention show increased signal magnitude and stability. In certain embodiments, modified luciferases of the invention show at least a 1, 2, 3, 4, 5, 10, 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000-fold increase in the magnitude of the signal over signals seen with wildtype luciferases.

Modified luciferases of the invention may be intracellular (i.e., not secreted), or they may be modified to be secreted. In further embodiments, modified luciferases of the invention are engineered to further express a secretory signal, general at the amino terminal end. In some embodiments, the secretory signal is a synthetic sequence. In specific embodiments, the synthetic sequence is MLLK VVFA IGCI WQA (SEQ ID NO: 7). In yet further embodiments, the secretory signal is any signal that can induce secretion of the encoded protein, including without limitation an interleukin-2 secretory signal and a chymotrypsinogen secretory signal.

Vargula Luciferases of the Invention

In some aspects, the present invention provides a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, which is a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used).

In further aspects, the present invention provides a modified *Vargula* luciferase that shows increased signal and stability. In certain embodiments, the modified *Vargula* luciferase of the invention is human codon optimized to increase expression in mammalian systems. In further

embodiments, a modified *Vargula* luciferase of the invention includes a wildtype or a native human codon optimized luciferase with the last two amino acids have been mutated CQ to SN (S=serine, N=asparagine). In still further embodiments, the present invention provides a mammalian vector expressing modified human codon optimized *Vargula* luciferase expressing intracellular *Vargula* luciferase. This sequence is the same as the wildtype or native human codon *Vargula* luciferase with the last two amino acids mutated CQ to SN (S=serine, N=asparagine) and with a KDEL (endoplasmic reticulum retention) sequence added after the C-terminal asparagine residue.

Firefly Luciferases of the Invention

In some aspects, the present invention provides a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm) and a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm).

In further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases. Such human codon optimized red-emitting *L. Italica* luciferases show significantly increased activity over wildtype red-emitting *L. Italica* luciferases (see FIG. 8). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases according to the sequence provided in FIG. 30 (SEQ ID NO: 3). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 3. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 3.

In still further embodiments, the present invention provides secreted red-*Italica* luciferases. FIG. 10 shows a comparison of luciferase activity of a human codon optimized red-emitting *L. Italica* luciferases fused to a chymotrypsinogen secretory signal to a non-secreted form of the human codon optimized red-emitting *L. Italica* luciferase. As discussed above, a number of different secretory signals can be used to produce secreted forms of modified luciferases of the invention. However, for red firefly luciferase, not all secretory signals produce a secreted luciferase. For example, popular signal sequences such as the N terminal 16 amino acid sequence of *Gaussia* luciferase and the Interleukin 2 secretory sequence do not successfully produce a secreted form of red emitting firefly luciferase.

Fusing a chymotrypsinogen secretory signal to a human codon optimized red-emitting *L. Italica* luciferases did successfully produce a secreted form of this luciferase. In some embodiments, the present invention provides a red firefly luciferase (also referred to herein as "red-emitting luciferase" and "red-emitting *L. Italica* luciferase") that is modified to include a synthetic secretory signal. In certain embodiments, the modified red firefly luciferase is encoded by the polynucleotide has the sequence provided in FIG. 4 (SEQ ID NO: 2). In still further embodiments, the present invention provides a luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 2. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 2. The underlined portion of FIG. 4 is the secretory signal. FIG. 11 shows a comparison of luciferase activities in supernatants and lysates of HEK293 cells transfected with a secreted red *Italica* Luciferase of the invention.

In further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases. Such human codon optimized green-emitting *L. Italica* luciferases show significantly increased activity over a previously described thermostable mutant of green-emitting *L. Italica* luciferase (B. R. Branchini et al., Analytical Biochemistry, 361 (2): 253-262 (2007)—see FIG. 9). In still further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases according to the sequence provided in FIG. 31 (SEQ ID NO: 4). In still further embodiments, the present invention provides human codon optimized sequences of luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 4. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 4.

Renilla Luciferases of the Invention

In some aspects, the present invention provides a modified efficiently secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc), which more stable than the wild-type *renilla reniformis* luciferase, and a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *Renilla* luciferase. Mammalian cells transfected with the secreted green *Renilla* luciferase mutant described here show approximately 35-fold higher luciferase activity compared to mammalian cells transfected with the native (human codon optimized) *Renilla* luciferase (see FIG. 7). Further the secreted green *Renilla* luciferase shows excellent stability of the bioluminescent signal (without compromising signal intensity) when assayed using the *Renilla* luciferase assay reagent described in this application (with the stabilizer included, see FIG. 7), thus making it an ideal reporter for High throughput screening applications.

In certain embodiments, the present invention provides a green-emitting *Renilla* luciferase plasmid sequence with the sequence pictured in FIG. 3 (SEQ ID NO: 1).

Gaussia Luciferases of the Invention

In some aspects, the present invention provides a *Gaussia* luciferase (emission max 482 nm) that is either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL). Such *Gaussia* luciferases can be used in single, double and triple reporter assays as discussed in further detail herein in combination with any of the other luciferases discussed herein or known in the art.

Luciferase Assays of the Invention

In certain aspects, the present invention provides compositions that improve stability and signal for assays utilizing wildtype and/or modified luciferases of the present invention.

In some embodiments, sodium chloride is added to improve the stability of luciferase assays of the invention. In such embodiments, a concentration of sodium chloride is utilized that improves the stability of the bioluminescent signal without affecting intensity. In further embodiments, sodium chloride concentrations in the range of about 0.05 M to about 1 M are used to improve stability of luciferase assays of the invention. In still further embodiments, sodium chloride concentrations of about 0.05 to about 0.5, 0.1 to about 0.4, about 0.2 to about 0.3, and about 0.05 to about 0.2M are used in luciferase assays of the invention. In specific embodiments, sodium chloride is added to improve the stability of assays utilizing wildtype and/or modified *Vargula* luciferases.

In further embodiments, certain luciferase substrates are added to luciferase assays to improve the stability of the bioluminescent signal. In such embodiments, the substrate added as a stabilizer may be an additional substrate that is not the substrate upon which the luciferase itself acts. For example, in assays utilizing *Cypridina* luciferase, coelenterazine is added to the assay to stabilize the assay stability. Coelenterazine is an oxidizable luciferin that is easily prone to oxidation but is not a substrate for the *Cypridina* luciferase. As will be appreciated, any luciferase assay described herein can be further modified by adding substrates for other luciferases as a stabilizer.

In some embodiments, the concentration of luciferase substrate is adjusted to improve the magnitude and/or stability of the signal. In further embodiments, low (under 1 μ M) concentrations of substrate is used to improve luciferase signals. For example, for *Cypridina* luciferase assays, about 1 to about 25 nM Vargulin are used in assays of the invention. In further embodiments, about 1-100, 5-90, 10-80, 15-70, 20-60, 25-50, and 30-40 nM Vargulin are used in assays of the invention. In further exemplary embodiments, substrates for the luciferase assays described herein (including *Cypridina*, *Gaussia* and *L. Italica* luciferases) are added in concentrations of from about 1 nM to about 250 μ M. In still further embodiments, substrates are added in concentration of about 10 nM-200 μ M, 50 nM-150 μ M, 100 nM-100 μ M, 150 nM-50 μ M, 200 nM-25 μ M, 300 nM-10 μ M, 500 nM-1 μ M.

In some embodiments, *Gaussia* luciferases of the invention are used with optimized reagents to produce increased activity. Kinetics of the *Gaussia* luciferase assay using the GAR-1 reagent is shown in FIG. 14. Measurement of the luciferase activity in supernatants of cells (transfected with *Gaussia* luciferase) using GAR-1 reagent from Targeting systems showed increased activity from *Renilla* luciferase assays from another vendor. The data in FIG. 14 is presented as an average of triplicate determinations measured on a Turner TD2020 luminometer. GAR-1 reagent has been described in detail in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay.

In certain embodiments, *Gaussia* luciferase assays of the invention utilize reagents stabilized with stabilizing agents. In one non-limiting example, the stabilizing agents include NP40 (Sigma) and/or coelenterazine. In certain embodiments, about 5 to about 200 μ M coelenterazine is used. In still further embodiments, about 10-150, 20-125, 30-100, 40-75, 50-60 μ M coelenterazine is used. In yet further embodiments, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 μ M coelenterazine is used. Stability of *Gaussia* luciferase assays using the GAR-2 reagent are shown in FIG. 15. Using the GAR-2B version of the *Gaussia* luciferase assay reagent, the bioluminescent signal remains very stable (FIG. 15A) In the absence of the stabilizer, the signal intensity is a little higher initially but decays faster than in the presence of the stabilizer (FIG. 15B). Note that the data presented in FIG. 15A and B is an average of triplicate determinations measured on a Turner TD2020 luminometer. The GAR-2 and GAR-2B reagents are stabilized versions of the GAR-1 reagent discussed in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to reagents for *Gaussia* luciferase assays. The GAR-2 reagent includes the composition GAR-1 with an additional 30 μ M coelenterazine. GAR-2B reagent includes the composition GAR-1 with and additional 75 μ M coelenterazine. Without being limited by theory, it is possible that the higher (approximately 3-fold) signal

intensity seen with the GAR-2B reagent is due to the higher concentration of coelenterazine. FIG. 12B shows the stability of the *Gaussia* luciferase with the GAR-2 reagent including a stabilizer.

In certain embodiments, stability of firefly luciferase assays is improved using FLAR-1 reagents (Targeting Systems). FIG. 16 shows the results from experiments using the FLAR-1 reagent from Targeting Systems. In the experiments shown in FIG. 16, the FLAR-1 reagent was added to the supernatant cell culture media.

Dual and Triple Luciferase Assays

In some aspects, the present invention provides dual luciferase assays based on spectral resolution of two or more different luciferases. As will be appreciated, these assays can include different wildtype luciferases, different modified luciferases, or a mixture of a wildtype and a modified luciferase. Such assays rely on differences in the emission spectra of the reporters used. In further embodiments, reagents are modified to allow for more efficient multiplexing. For example, when *Gaussia* luciferases are multiplexed with firefly luciferases, EDTA is omitted from the reaction mixture to allow efficient reporter activity.

FIG. 13A shows the emission spectra of a dual reporter assay utilizing a *Vargula* and Red *Italica* luciferase of the invention. The luciferases were expressed in samples of transfected cells. The luciferases used in the experiments pictured in FIG. 13A represent a modified red emitting firefly luciferase of the invention that is human codon optimized and intracellular (non-secreted) and a *Cypridina* luciferase of the invention that is from *Cypridina hilgendorfi* modified to be human codon optimized and secreted.

FIG. 13B shows the emission spectra of a triple reporter assay utilizing *Vargula*, Green *Renilla* and Red *Italica* luciferases. These emission spectra were in samples of transfected cell lysates. The *Vargula* and red-emitting firefly luciferases are those as described above for FIG. 13A and the Green *Renilla* luciferase is an improved secreted Green luciferase mutant as described in further detail herein.

All patents and other references cited in the specification are indicative of the level of skill of those skilled in the art to which the invention pertains, and are incorporated by reference in their entireties, including any tables and figures, to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to obtain the ends and advantages mentioned, as well as those inherent therein. The methods, variances, and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention

that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Also, unless indicated to the contrary, where various numerical values or value range endpoints are provided for embodiments, additional embodiments are described by taking any 2 different values as the endpoints of a range or by taking two different range endpoints from specified ranges as the endpoints of an additional range. Such ranges are also within the scope of the described invention. Further, specification of a numerical range including values greater than one includes specific description of each integer value within that range.

Thus, additional embodiments are within the scope of the invention and within the following claims.

EXAMPLES

Example 1

Transfection of Mammalian Cells with Modified Luciferases

HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing either the human codon-optimized or non-human codon optimized forms of the red emitting and green emitting firefly luciferases (from *Luciola Italica*) under control of the CMV promoter. Transfections were performed using the Targefect F-2 reagent (Targeting Systems) using the manufacturers protocols. Forty eight hours post transfection, the cells were lysed using the cell lysis reagent (CLR-1) from Targeting Systems, Santee. 20 μ l aliquots of the cell lysate were mixed with 100 μ l of the FLAR-1 (firefly luciferase assay reagent from Targeting Systems).

Example 2

Cypridina Luciferase Assays with Increased Stability

Compositions were developed for achieving optimal performance of *Cypridina* luciferase assay reagents. These assays had improved stability of the bioluminescent signal without affecting the overall activity of the enzyme.

Vargulin is generally unstable and easily oxidized, making long term storage of this substrate difficult. However, Vargulin stored in an acidic buffer (66 mM monobasic potassium phosphate, pH 6-6.5) and stored at -80° C. was very stable and did not lose activity even when stored for several months. In contrast, Vargulin dissolved in a neutral to basic phosphate buffer (e.g. 200 mM dibasic potassium phosphate (pH 8)) is very unstable and begins to lose activity rapidly within a few hours at room temperature. *Cypridina* luciferase activity was

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optimal when 200 mM dibasic potassium phosphate was used as the reaction buffer instead of 66 mM monobasic sodium phosphate. Hence 200 mM dibasic potassium phosphate was used as the reaction buffer. Concentrations of be 3-6 mM Vargulin were found to be effective, and these concentrations are much lower than what is generally used in such assays (see for example Wu et al (2007) *Biotechniques*, 42(3):290-292).

The *Cypridina* luciferase assay showed increased stability when sodium chloride was included in the reaction. For example, FIG. 1 shows the relative luciferase stability (RLS) between VLAR-1 (no sodium chloride) and VLAR-2 (VLAR-1+sodium chloride). Sodium chloride clearly stabilized the RLS. For the experiments in FIG. 1, 20 μ l of sample was added with 40 μ l of VLAR solution for the assay followed by 20 μ l of Vargulin substrate.

FIG. 5 shows a further titration experiment indicating that sodium chloride concentrations of around 0.5M provide increased stability over control reagents with no sodium. Further concentrations that are of use in stabilizing such assays include from about 25 mM to about 750 mM sodium chloride. For experiments in FIG. 5, 5 μ l of the indicated concentrations of sodium chloride solutions were added to 35 μ l of VLAR buffer (20 mM dibasic potassium phosphate, pH=8.0). The assay was carried out by mixing 20 μ l of sample with 40 μ l of VLAR buffer (with sodium chloride) and then adding 20 μ l of *Cypridina* luciferin.

Further stability of the bioluminescent signal as well as improvement in overall luciferase activity was observed when coelenterazine, another oxidizable luciferin easily prone to oxidation (but not a substrate for *Cypridina* luciferase) was included in the assay composition. A 15 minute pre-incubation was found to result in increased stability of the bioluminescent signal using sample volumes between 5 and 20 μ l (roughly 40% drop in 26 minutes using an assay volume of 20 μ l and 15% drop in 26 minutes using an assay volume of 5 μ l—see FIGS. 2A and 2B. A concentration of coelenterazine that worked well to stabilize the reagent was 15 μ M. Concentrations in the range of about 10 μ M to about 50 μ M can also be used. The inclusion of coelenterazine in the composition decreased the background of the assay by more than 10-fold (background reading dropped from 153.6 to 12.4) and also resulted in a 15% increase in the intensity of the bioluminescent signal. Controls in which buffers with identical composition (i.e., inclusion of coelenterazine but omission of *Cypridina* luciferin) showed no activity. Coelenterazine is not a substrate for *Cypridina* luciferase and can be used to safely reduce the background and increase stability when *Cypridina* luciferase is assayed alone or in combination with other luciferases (such as firefly luciferase) which do not use coelenterazine as a substrate. For the experiments shown in FIG. 2, 5 or 20 μ l of the sample (media supernatant) was mixed with 40 μ l of the VLAR buffer (200 mM dibasic potassium phosphate, 50 mM NaCl). The firefly and *Cypridina* luciferase assay reagents can be mixed into a single solution which can be used to efficiently measure both *Cypridina* luciferase and firefly luciferase activity by spectrally resolving the luciferases using appropriate filters. However, the DTT concentration in the firefly luciferase assay reagent can affect activity in such situations, because the activity of both luciferases is decreased due to interference of DTT (present in low concentration in the firefly assay reagent with the *Cypridina* luciferase assay (there is almost a 10-fold drop in *Cypridina* luciferase activity). However, since the signal intensity of the *Cypridina* luciferase assay is very robust, the signal is still acceptable and improvement in *Cypridina* luciferase activity is observed if the DTT concentration in the

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firefly luciferase assay reagent is dropped to 2.5 mM (a 3 fold drop in activity of *Cypridina* luciferase is still observed). Single solution based dual assays in which *Cypridina* luciferase is multiplexed with Green emitting *Renilla* luciferase work very well without loss of activity of either *Cypridina* or *renilla* luciferase when the two solutions are mixed.

Example 3

Renilla Luciferase Assays Utilizing Modified *Renilla* Luciferases and Stabilizing Reagents

The secreted modified green *Renilla* luciferase of the present invention showed significantly greater activity over wildtype *Renilla* luciferase—see FIG. 7. For the experiments pictured in FIG. 7, HEK 293 cells were transfected with expression vectors expressing either native *Renilla* luciferase or the secreted Green *Renilla* luciferase mutant. Cells were lysed 48 hrs post transfection and assayed for luciferase activity.

Assays with and without stability assay reagents for green *Renilla* luciferase were investigated. FIG. 6 shows that assays conducted with stabilizer showed greater stability than those without. The composition of the *Renilla* luciferase assay reagent (no stabilizer) was: 30 μ M coelenterazine, 0.4 \times PBS (Ca, Mg free), 0.027% NP40. The composition of the *Renilla* luciferase assay reagent (with stabilizer) was: 30 μ M coelenterazine, 0.4 \times PBS (Ca, Mg free), 0.227% NP40. Stabilizer is 2% NP40 (a non-ionic detergent).

Example 4

Kinetics of Different Luciferases

Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the luciferase assay reagents supplied with the LiveResponse assay kit. These data are shown in FIG. 12A: Red *Luciola* (firefly), luciferase, FIG. 12B *Gaussia Princeps* luciferase (this is FIG. 15C), FIG. 12C: *Cypridina* luciferase, and FIG. 12D: Green *Renilla* luciferase. Data represents mean of triplicate determinations.

Example 5

Comparison of Expression Vectors Expressing Modified *Vargula* Luciferases

Transfection protocols were as follows: HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing either the human codon-optimized to non-human codon optimized forms of native VLuc, HC-VLuc, sequence 1) or modified HC-VLucs under control of the CMV promoter. Transfections were performed using the Targefect F-2 reagent (Targeting Systems) using the manufacturers protocols.

The stability of the bioluminescent signal of *Cypridina* Luciferase assessed using supernatants of HEK293 cells transiently transfected with the pCMV VLuc expression vector is shown in FIG. 17.

In FIG. 19, the stability of the bioluminescent signal of *Cypridina* Luciferase was assessed using supernatants from HEK 293 cells transiently transfected with the pCMV-VLuc expression vector. Samples were assayed using the VLAR-2

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(VLAR-1 reagent from Targeting Systems with sodium chloride) of the *Cypridina* luciferase assay reagent.

Human codon optimization of the gene sequence encoding the VLuc led to a 5-fold improvement of luciferase expression in HEK-293 transfected with expression vectors containing the human codon optimized versions of the *vargula* luciferase genes compared to the native sequences (i.e. Non-human codon optimized sequences). Addition of the KDEL sequence at the C-terminal end results in intracellular expression of VLuc.

Example 6

Construction of Blue-Emitting (Blue Shifted) and Green Emitting Mutants of Secreted *Renilla* Luciferase for Use as Secreted Reporters in Single or Multiplexed Luciferase Assays

A synthetic signal peptide was deduced by rational design: MLLK VVFA IGCI VVQA (SEQ ID NO: 7). The sequence of this signal peptide was based on rational design using signal sequences from the secretory signals known in the art, including those available at: <http://www.unitargeting.com/Resources/Trends07.pdf>

Blue-Shifted Secreted *Renilla* Luciferase Mutants

Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Loma Linda paper, cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *Renilla* luciferase. MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M. Emission maxima=475 nm

Secreted BLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal (Emission Maxima 475 nm)

Red Shifted *Renilla* Luciferase Mutants:

Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A, D162E

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

Example 7

Tests for Developing Assays for *Vargula* Luciferase

In some embodiments, different buffer solutions are used to improve assays utilizing wildtype and/or modified luciferases of the invention. In certain embodiments, a 1:1 mixture of 0.1 M Tris HCl and 75 mM sodium phosphate is used as the assay buffer.

Several different parameters were tested to develop an assay for *vargula* luciferase:

Effects of using either an acidic buffer (e.g., potassium phosphate pH 5-6.8), Tris HCl pH 7.4, Tris phosphate buffer pH (8-8.5) as well as varying assay volumes were tested. In

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general the use of acidic conditions significantly reduced the intensity of the bioluminescent signal (typically 5-10 fold) while increasing the stability somewhat. Using Tris HCl pH 7.4, the activity as the assay buffer resulted in 5-10 fold brighter bioluminescence but the luminescent signal was highly unstable.

Use of a buffer mixture (1:1) of 50 mM Tris HCl, pH 7.4 and 100 mM dibasic sodium phosphate resulted in improved stability of the bioluminescent signal without compromising the intensity of the bioluminescent signal. An interesting finding was that inclusion of 0.2 M NaCl further increased stability of the bioluminescent signal. Lastly the amounts of Vargulin needed for optimal activity using this buffered condition are very low (1-10 nM range) making the assay extremely useful and economical.

Increasing the concentration of Vargulin further did not increase stability of the assay further.

Stock Vargulin substrate solutions stored in an acidic condition pH (5.5-6) were relatively stable over several months when stored at -80° C.

Other parameters tested: Other stabilizers such as DTT (dithiothreitol), detergents like NP-40 or EDTA were unable to increase the intensity of the luminescent signal or improve stability of the assay. EDTA decreased the VLuc activity by at least 5-fold.

Thus one aspect of the invention concerns the following composition and variations thereof: 20 µl of cell supernatant assays with 50 µl of Tris/phosphate buffer, pH 8, 0.2 M NaCl, 10 µl of 5-100 nM vargulin in 66 mM potassium phosphate (monobasic). In certain assays, the effective concentration of vargulin in the assay mix is as low as 20 nM which is approximately 50-fold lower than that reported in the literature (see for example Wu et al (2007) Biotechniques, 42(3):290-292)

Comparison of luciferase activity in cells transfected with *vargula* luciferase with luciferase activity in cells transfected with firefly luciferases from *Photinus pyralis* or *Luciola Italica* showed that *vargula* luciferase was a much more sensitive reporter (10-20 fold improvement in bioluminescent signal compared to firefly luciferase, assay done in HEK-293 cells, all expression vectors were expressed luciferase under control of the CMV promoter). An exemplary assay protocol included: 20 µl aliquots of Cell supernatants (media with 5% serum) were mixed with 100 µl of assay dilution buffer (50 µl of 50 mM TrisHCl, 100 mM dibasic sodium phosphate, pH 8) and 10 µl of vargulin in sodium phosphate buffer pH 6 (final concentration of vargulin in reaction mix 10-25 nM). The sample was mixed well and bioluminescent activity was recorded in a Turner TD2020 luminometer integrated over a 20 sec time interval.

Example 8

Activity in Cell Supernatant and Cell Lysates of Cell Transfected with Either a Plasmid Vector Expressing Secreted *Vargula* Luciferase or an Intracellular Form of *Vargula* Luciferase

In cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity was secreted into the cell supernatant and only 20% is cell-associated.

FIG. 18 shows intracellular and secreted *Cypridina* luciferase activity. Luciferase activity in cell supernatants and cell lysates of cells transfected with a plasmid vector expressing secreted *vargula* luciferase. As shown in FIG. 18 cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity is secreted into the cell supernatant and only 20% is cell-associated.

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In cells transfected with *vargula* luciferase modified at the C-terminal end with a KDEL sequence, approximately 95% of the activity was intracellular and 5% is secreted.

Example 8

Development of a Dual Reporter System Based on Blue and Red Shifted Mutants of Secreted *Renilla* Luciferase

Secreted mutants: Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *renilla* luciferase: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; N53Q; V146M. Emission maxima=475 nm

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations. C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal. Emission Maxima 475 nm

RED SHIFTED *RENILLA* LUCIFERASE MUTANTS: Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A, D162E

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 4: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations. A123S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

A single solution dual luciferase assay based on secreted *renilla* luciferase blue emitting (emission max at 475 nm) and green emitting mutants (emission max at 535 nm).

The mutations in the above sequences lead to the efficient expression of secreted *renilla* luciferase in the transfected cells. The two luciferases can therefore be used in combination as a dual reporter system and the luciferase activity of each luciferase in the transfected cells can be resolved by using appropriate filters. The reagent compositions for *renilla* luciferase assay reagents are described Walia, US Pat Appl Publ 2008074485, entitled Enhancing a Luminescent Signal, which is incorporated herein by reference in its entirety and in particular for all teachings related to *Renilla* luciferase assay reagents.

Example 9

Development of a Triple Reporter System Based on Red and Green Emitting Firefly Luciferases and *Gaussia* Luciferase/*Renilla* Luciferase

Composition of the *Gaussia* luciferase assay reagent (GAR-1) has been described in detail in a US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay. An assay reagent useful for simultaneous measurement of all three reporters in a single solution was designed by omitting EDTA from the composition of the *Gaussia* luciferase assay reagent and then including all the ingredients necessary for assay of firefly

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luciferase in a single composition. The rationale behind this is that the EDTA interferes with the firefly luciferase assay (magnesium is an important co-factor for firefly luciferase and EDTA chelates magnesium). The ingredients required for Firefly luciferase assay included in the assay composition were as follows—ATP, DTT. Firefly luciferin, magnesium sulfate, magnesium bromide (helps increase brightness of luminescent signal) and phosphate buffer.

The composition of the single solution for a triple reporter assay for measuring *Gaussia* luciferase or *Renilla* luciferase in combination with red and green emitting firefly luciferase is as follows:

0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:

To 800 ml of the above solution add the following:

Tricine 3.227 g (20 mM)

1M Magnesium sulfate 0.7H₂O 2.51 ml (2.67 mM)

Magnesium bromide 0.6 H₂ (1.07 mM)—add 2.14 ml of 500 mM stock solution

25 mM OTT (3.86 g)

530 μM ATP (2.72 g)

CoA (0.18 g)—optional

Adjust with sodium phosphate to pH 7.8

Add 940 μM D-Luciferin (free acid)—253.81 mg

CDTA-0.8289 g

940 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

0.8M Tris (0.02 M EDTA)—43.53 ml

Add GAR reagent without EDTA to a total volume of 1 liter

Dilute 100× coelenterazine substrate with the above solution to 1× just before use. Use normal 3 mg/5 ml absolute alcohol acidified with 30 μl of 2N HCl)

NOTE: This assay reagent does not contain enough cell lysis reagents. Hence cells have to be first lysed using 1× Cell Lysis Buffer (compatible with use of all luciferases (prepared from 5× stock solution described below:

Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× Cell Lysis Buffer:

For 1 liter of Buffer

5 ml NP 40 (undiluted)

25 ml Tris HCl pH 8

1.45 g NaCl

50 ml glycerol

Example 10

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

A *vargula* luciferase-based triple reporter system was prepared by first preparing the *vargula* luciferase assay reagent (VLAR-1) and mixing it in a 1:1 ratio with the firefly luciferase assay reagent (FLAR-T) to give the triple assay reagent TVLAR-1.

Assay protocol: To 20 μl of cell lysate add 100 μl of the TVLAR-1 reagent and read in the Victor luminometer (Perkin Elmer) or Varian (Promega) using appropriate filters.

Preparation of VLAR-1 Reagent:

Composition of the *Vargula* Luciferase Assay reagent is described below

500 ML OF 0.1 M TRIS HCL PH 8

500 ML of dibasic sodium phosphate 200 mM

200 ml of 5 nM Vargulin in 66 mM potassium phosphate pH 5.5

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pH of final solution is 8-8.5
 Composition of the FLAR-T Reagent
 SOLUTION A: 0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:
 To 800 ml of the above solution add the following:
 Tricine 3.227 g (20 mM)
 1M Magnesium sulfate. 7H₂O 2.51 ml (2.67 mM)
 Magnesium bromide (0.6 H₂) (1.07 mM)—add 2.14 ml of 500 mM stock solution
 5 mM DTT (in some embodiments, any range between 5 mM and 30 mM can be used, including 5, 10, 15, 20, 25, 26, 27, 28, 29, and 30 mM)
 530 μM ATP (2.72 g)
 CoA (0.18 g)—optionally omitted
 Adjust with sodium phosphate to pH 7.8
 Add 940 μM D-Luciferin (free acid)—253.81 mg
 CDTA-0.8289 g
 940 μM D-luciferin (free acid)—253.81 mg
 CDTA-0.8289 g
 941 μM D-luciferin (free acid)—253.81 mg
 CDTA-0.8289 g
 0.8M Tris (0.02 M EDTA)—43.53 ml

ADD SOLUTION A to a total volume of 1 liter

NOTE: This assay reagent does not contain enough cell lysis reagents for effective lysis. Hence cells should first be lysed, e.g., using 1× Cell Lys is Buffer (compatible with use of all luciferases (prepared from 1× stock solution described below: Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× cell lysis buffer:

For 1 liter of Buffer
 5 ml NP 40 (undiluted)
 25 ml Tris HCl pH 8
 1.45 g NaCl
 50 ml glycerol

Composition of Firefly luciferase assay reagent (for use of firefly luciferase as a single reporter gene).

20 mM tricine (179.2 g)
 MgCo₃ 1.07 mM 0.55 g
 Magnesium sulfate 2.7 mM (277 ml)
 0.1 mM EDTA
 20 mM DTT (4.25 g)
 530 μM ATP (3 g)
 CoA (0.198 g)
 Add disodium phosphate 25 g to pH 7.8
 Add 793 ml water before pH
 470 μM D Luciferin free acid 279.2 mg
 5×CCLR 307 ml
 Composition of 5×CCLR:
 0.8 M Tris 0.02 M EDTA pH 8-156 ml
 Glycerol 500 ml
 Triton X100 50 ml
 CDTA-7.5 m moles (2.7 g)
 DTT 10 mM 1.542 g total vol 1 liter.

Example 11

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

Addition of stabilizer does not significantly affect (i.e., there is very little decrease in signal intensity) intensity of bioluminescent signal of *Renilla* luciferase in supernatants and lysates. FIG. 20 (top panel) shows a *Renilla* assay performed with 10 μl of *Renilla* Lysate and 20 μl of *Renilla*

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Supernatant. Assay went as follows: 20 or 10 μl of sample (Supernatant or Lysate), 50 μl of RLAR-1 reagent (Targeting Systems). FIG. 20 (bottom panel) shows *Renilla* Assay was performed using the same volumes of lysate and supernatant as in the experiments in the top panel. Assay protocol was as follows: 10 or 20 μl of lysate or supernatant depending on the assay, 50 μl of the RLAR-1 reagent and an additional 8 μl of RLAR stabilizer for an increased stability profile for a time course reading. The stabilizer lowered the initial RLU reading (decreased from approximately 9000 to approximately 7000 rlu) but showed a much higher level of stability when observed over 30 minutes to 1 hour (FIG. 12C). The RLAR-1 reagent is useful for high throughput screening (HTS) applications in which a large number of samples need to be assayed. In the absence of the stabilizer, the signal intensity decays faster than in the presence of stabilizer (FIG. 21). Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. In FIG. 21, a time course was taken using the standard protocol of 10 μl lysate, 50 μl of RLAR reagent without stabilizer indicating drop in *Renilla* luciferase activity.

FIG. 22 shows the stability of the bioluminescent signal of *Cypridina* luciferase and firefly luciferase using the DLAR-3 reagent. This reagent is useful for HTS applications involving both *Cypridina* luciferase and the red-emitting *Luciola* luciferase. Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. The DLAR-3 reagent (Targeting Systems) is a dual assay reagent based on secreted *Cypridina* luciferase and a secreted or intracellular red-emitting firefly luciferase.

FIG. 28 shows emission spectra of *Cypridina* and Firefly luciferases in samples of transfected cells (lysates or supernatants). The emission spectra were recorded on a Fluorolog-3 spectrofluorometer (Horiba Scientific, Japan) using a liquid nitrogen cooled CCD. The luciferases were assayed by mixing 200 ul of the sample with the appropriate luciferase assay reagent to obtain spectral profiles. Emission max of *Cypridina* Luciferase is 463 nm; Red *Italica* 617 nm.

Example 12

Double and Triple Luciferase Reporter Assays Based on *Renilla* Luciferase, Firefly Luciferase and *Vargula* Luciferase

Kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the DLAR-5 system are shown in FIG. 23. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the DLAR-5 luciferase assay reagents. The decay of the *renilla* luciferase signal shown in Panel B above can be greatly minimized (ie the bioluminescent signal can be rendered much more stable by addition of a *Renilla* luciferase stabilizer to the DLAR-5 buffer.

FIG. 24 shows Emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activities of *Cypridina*, Green *Renilla* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm. Note that the data presented in this application is performed with the green-emitting mutant that emits at 527 to 530 nm (this is the variation in emission maxima seen and the luciferase is different in sequence, properties and emission maximum from the 535 nm emitting intracellular green emitting *Renilla* luciferase mutant described in US Patent Publication No.

20090136998, which is hereby incorporated by reference in its entirety and in particular for all teachings related to Green *Renilla* luciferase.

FIG. 25 shows kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the triple reporter system. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were measured using the TLAR luciferase assay reagents (Targeting Systems).

FIG. 26 shows emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activi-

ties of *Cypridina*, *Renilla* and Red *Luciola Italia* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm and Red *Luciola Italia* luciferase is 617 nm.

The present invention also provides a single solution-based triple luciferase reporter assay involving *Cypridina* luciferase multiplexed with Green-emitting *Renilla* luciferase and Red-emitting Firefly luciferase. This assay is compatible with high throughput applications. This assay is also optionally in a format where the three luciferases can be assayed separately using three different assay reagents

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What is claimed is:

1. A multiplexed luciferase assay composition comprising: multiple luciferase reporters, wherein at least two of the luciferase reporters are selected from the group consisting of a firefly luciferase, a *Renilla* luciferase, a *Gaussia* luciferase and a *Cypridina* (*Vargula*) luciferase, and wherein the different luciferase reporters emit at different wavelengths and/or utilize different substrates, and

60 wherein the firefly luciferase is a red-emitting human codon optimized luciferase encoded by SEQ ID NO:3 with an emission maximum of approximately 617 nm; or
65 wherein the firefly luciferase is a green-emitting human codon optimized luciferase encoded by SEQ ID NO:4 with an emission maximum of approximately 550 nm; or

57

wherein the *Renilla* luciferase comprises A55T, S130A, K136R, A143M, M185V, M253L, and S287L mutations or A123S, D154M, E155G, D162E, I163L, and V185L mutations compared to wildtype *Renilla* luciferase; or wherein the *Vargula* luciferase is encoded by SEQ ID NO: 5 6; and wherein at least one of the luciferase reporters comprises a secretory signal, wherein the secretory signal is peptide sequence of SEQ ID NO: 7.

* * * * *

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58